THE INTEGRATION OF ADENOVIRUS DNA

DURING PRODUCTIVE AND ABORTIVE INFECTION

BY

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STATEMENT

The experiments referred to as: 'Limitations of the network method' in Chapter 2, and as: 'The integration of the DNA of Ad5 ts36 and Ad5 ts125 in human embryo kidney cells (HEK)' in Chapter 3, were done in collaboration with Dr A.J.D. Bellett. The rest of the work is my own.

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ABSTRACT

The work described in this thesis aims to improve understanding of the event of integration of viral DNA during infection with adenoviruses. Evidence for the occurrence of integration of adenovirus DNA during productive and abortive infection is reported.

The integration of adenovirus DNA during productive infection was investigated with CELO virus, and avian adenovirus, in Chick Embryo Kidney (CEK) cells. Cell DNA from CELO virus infected CEK cells was separated from free viral DNA by three different methods. The cell DNA was then shown to contain viral DNA sequences by reannealing kinetics. Uninfected cells to which a known amount of viral DNA was added, were used as a control. Most integration occurred at late times in infection.

Human cells (HEK) were infected by DNA negative mutants ts125 or ts36 of Ad5. Both integrated their DNA in the absence of viral DNA replication. Ad5 ts36 does not transform abortively infected cells at the non permissive temperature, while Ad5 ts125 transforms them with higher frequency than wild type virus at both permissive and non-permissive temperature. In mouse cells abortively infected by Ad5 wild type or its mutants, the same percentage of viral DNA was integrated at either temperature. Each portion of the viral genome was integrated with equal frequency. The failure of Ad5 ts36 to transform at the non-permissive temperature is therefore not due to a failure of the viral DNA to integrate. Similarly, the increased frequency of transformation by Ad5 ts125 is not due to an increased capacity of the DNA to integrate.

CONTENTS

PUBLICATIONS

Most of the work reported in this thesis has been published or is going to be submitted for publication. These papers are:

Tyndall, C., H.B. Younghusband, and A.J.D. Bellett. 1978. Some adenovirus DNA is associated with the DNA of permissive cells during productive or restricted growth. J.Virol. 25:1.

Younghusband, H.B., C. Tyndall, and A.J.D. Bellett. 1978. Replication and interaction of viral and cellular DNA in mouse cells abortively infected by human adenovirus. To be submitted for publication.

Chapter 1

GENERAL INTRODUCTION:

A Literature Review

SCOPE OF THE REVIEW

This review is designed to introduce the reader to some aspects of viral DNA integration. It is restricted to viruses of either bacterial or animal origin which have been shown to insert their DNA into the genome of the host cell, i.e., to integrate. Emphasis is placed on discussion of animal DNA viruses, as particularly relevant to the work reported in Chapters 2 and 3.

INTRODUCTION

The term integration is rigorously defined as insertion of viral DNA by covalent bonds into host cell DNA following viral infection, and should be differentiated from terms like association of viral DNA with infected host DNA or persistence of viral DNA within the cell. Integration occurs during infection of the appropriate cell with bacterial or animal viruses. The best studied and most definitely established examples of integrated viral genomes are those of the temperate bacteriophages lambda, P2, P22 and Mu. Among animal viruses, integration of viral DNA has been shown to occur in DNA and RNA tumour viruses. The latter finding has elicited a great deal of interest in the phenomenon of integration with a view to understanding its role in transformation and oncogenesis. This survey, though emphasising the integration of the DNA of animal viruses, will also present selected topics dealing with lysogeny and temperate bacteriophages. A combined discussion of integration of viral DNA in prokaryotic and eukaryotic cells may be useful to improve our understanding of the basic mechanisms involved in integration by comparing and contrasting the events observed in different systems.

TEMPERATE BACTERIOPHAGES AND LYSOGENIC CELLS

After infection of a sensitive bacterial strain with temperate bacteriophages such as lambda, P22, P2 and Mu, two alternative responses are seen. Some cells are lysed by a process of phage multiplication identical to that of lytic phages, other cells are lysogenised, giving rise to normally growing cultures in which each cell harbours the phage in a non-infectious form. In the lysogenic state the phage DNA becomes integrated into the host DNA with concomitant repression of most of the viral functions; the viral DNA then replicates in the integrated form synchronously with the host chromosome. Three steps characterise the lysogenisation of a cell: establishment of lysogeny, maintenance of lysogeny and capacity for induction. All of these functions are encoded in and expressed by the viral genome. The establishment of lysogeny requires expression of the early genes necessary for the integration event and at the same time repression of the late genes responsible for lytic development. Maintenance of lysogeny is dependent on the maintenance of repression; induction of lytic development requires release of repression, excision of the viral genome and expression of its late genes. All these aspects of lysogeny will now be examined in more detail in the case of the best analysed temperate phages lambda and Mu, which present in some respects markedly different types of behaviour.

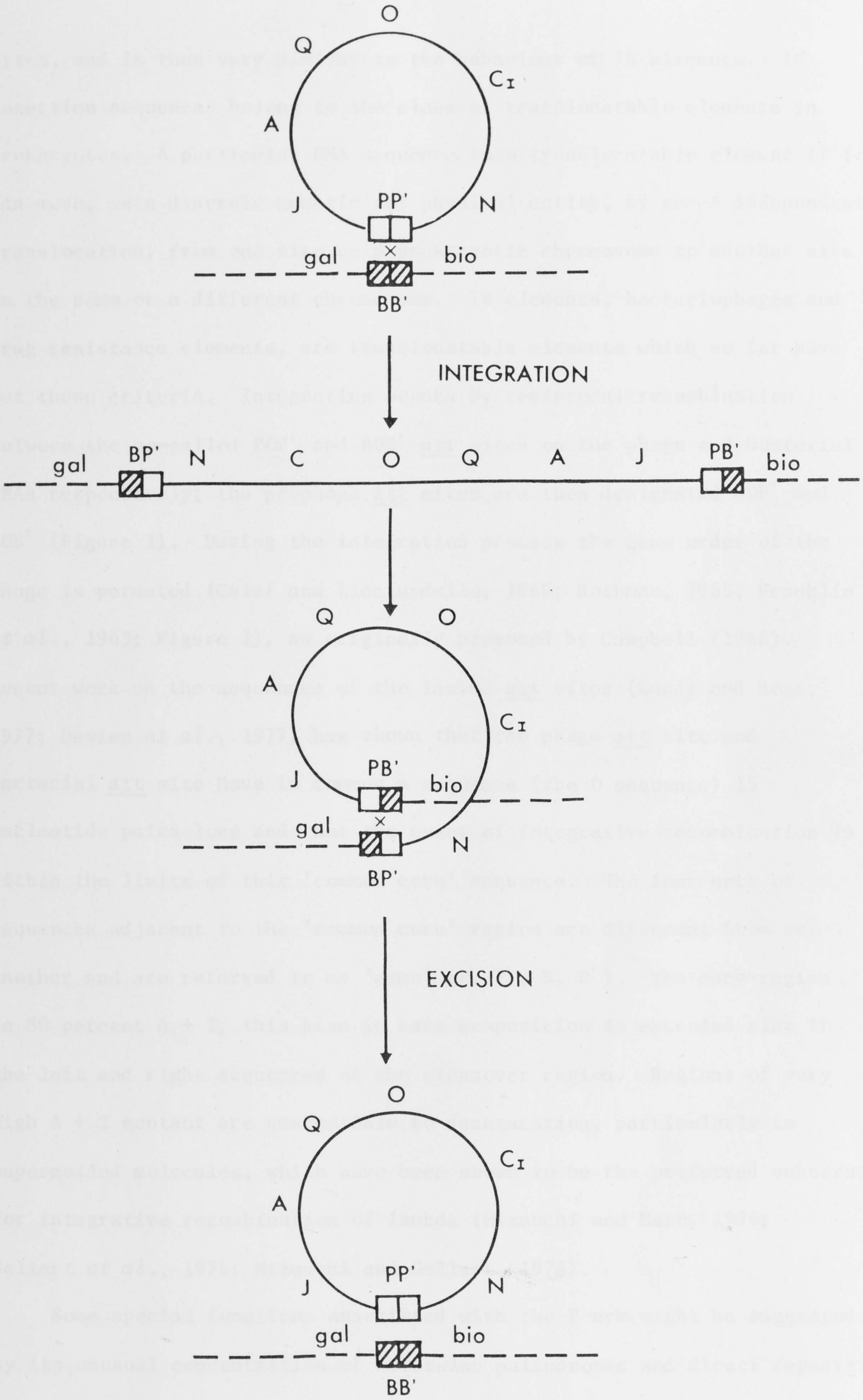
The lysogenisation by bacteriophage lambda (λ) of *Escherichia coli* K12 cells, which are sensitive to this phage constitutes an important model for the study of lysogeny. The DNA of bacteriophage lambda is a double stranded linear molecule of molecular weight 31×10^6 (Burgi and Hershey, 1963). It has cohesive ends (Hershey *et al.*, 1963), i.e., single stranded complementary sequences, 12 nucleotides long (Wu and Taylor, 1971) on either 5' terminus of the molecule.

The cohesive ends allow the molecule to circularise by means of hydrogen bonds. Upon infection of *E. coli* with lambda, the viral DNA is covalently closed by a DNA ligase (Young and Sinsheimer, 1964; Bode and Kaiser, 1965; Dove and Weigle, 1965; Gellert, 1967). During lysogeny, according to the now well documented model proposed by Campbell (Figure 1) the infecting phage DNA, after circularization, undergoes a single reciprocal recombination event at specific loci (att. sites) on the bacterial and viral chromosomes (Campbell, 1962). Repression of late viral functions is necessary for the establishment of lysogeny and is effected by the continuous expression of a repressor protein coded by the C_I gene (Ptashne, 1967). The expression of gene C_I is positively regulated by C_{II} and C_{III} gene functions (Reichardt and Kaiser, 1971; Echols and Green, 1971; Reichardt, 1975). The cro gene product turns off repressor synthesis by directly reducing C_{II} and C_{III} gene expression (Reichardt, 1975). Integration of the phage DNA is promoted by the product of the int gene, which maps immediately to the right of the att site. The int gene product is required for both integration and excision of the λ prophage (Zissler, 1967; Gingery and Echols, 1967; Gottesman and Yarmolinsky, 1968). The excision reaction requires, in addition, the product of a second phage gene, xis, which maps immediately to the right of the int gene (Guarneros and Echols, 1973; Kaiser and Masuda, 1973).

Lambda DNA is normally integrated between the gal and the bio sites on the host chromosome by alkali stable, and presumably covalent, bonds (Friefelder and Meselson, 1970). When *E. coli* chromosome carries deletion of the gal-bio region, that is, of the primary bacterial att site, int dependent integration of lambda can be detected at numerous loci (secondary bacterial att sites) on the *E. coli* chromosome though occurring at only about 0.5 percent of the normal frequency of integration (Shimada *et al.*, 1972; 1975). This integration always involves the phage att

FIGURE 1: Campbell's (1962) model for integration and excision of bacteriophage lambda

Rectangles represent the attachment sites on bacteriophage (continuous line) and cellular (broken line) DNAs. The attachment sites are represented formally as PP' on the phage DNA, and as BB' on the cellular DNA, to indicate that each of them is made up of non homologous recognition elements bracketing a central crossover point or region.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sites, and is thus very similar to the behaviour of IS elements. IS insertion sequences belong to the class of translocatable elements in prokaryotes. A particular DNA sequence is a translocatable element if it can move, as a discrete genetic and physical entity, by rec-A independent translocation, from one site on a prokaryotic chromosome to another site on the same or a different chromosome. IS elements, bacteriophages and drug resistance elements, are translocatable elements which so far have met these criteria. Integration occurs by reciprocal recombination between the so-called POP' and BOB' att sites on the phage and bacterial DNAs respectively; the prophage att sites are then designated BOP' and POB' (Figure 1). During the integration process the gene order of the phage is permuted (Calef and Licciardello, 1960; Rothman, 1965; Franklin *et al.*, 1965; Figure 1), as originally proposed by Campbell (1962). Recent work on the sequences of the lambda att sites (Landy and Ross, 1977; Davies *et al.*, 1977) has shown that the phage att site and bacterial att site have in common a sequence (the O sequence) 15 nucleotide pairs long and that the event of integrative recombination is within the limits of this 'common core' sequence. The four sets of sequences adjacent to the 'common core' region are different from one another and are referred to as 'arms' (P, P', B, B'). The core region is 80 percent A + T; this bias in base composition is extended also to the left and right sequences of the crossover region. Regions of very high A + T content are susceptible to denaturation, particularly in supercoiled molecules, which have been shown to be the preferred substrate for integrative recombination of lambda (Mizuuchi and Nash, 1976; Gellert *et al.*, 1976; Mizuuchi and Gellert, 1978).

Some special functions associated with the P arm might be suggested by its unusual concentration of molecular palindromes and direct repeats. The bacterial att site is by comparison sparse in such sequence features

and a less 'active' role seems consistent with the higher frequency - and implied lack of specificity - of secondary bacterial att sites. Two other possibly interesting features are associated with the att site sequences. One is the occurrence in the OP' core-arm junction of potential RNA polymerase recognition sites; the other is a homology between sequences at the core-arm OP' junction and the 3' end of 16S ribosomal RNA.

Finally, in and around the crossover region occur sequences that are also found close to the integration site of the DNA insertion elements IS 1 (Landy and Ross, 1977). This homology raises an interesting question about the relationship between the int protein and the proteins acting on IS 1, and the involvement of these sequences in recognition by host proteins. This sequence homology might also be related to the observation that at least one secondary insertion site for lambda on the *E. coli* chromosome is genetically inseparable from a site frequently occupied by IS sequence (Shimada *et al.*, 1973).

The functional significance of these sequences as well as that of the others mentioned above still remains to be shown and is at this stage only a matter for speculation. However, the DNA sequences presented by Wayne *et al.*, (1977) and Landy and Ross (1977) will provide the basis for further definition of important features of DNA sequences involved in site specific recombination.

Excision of the lambda DNA from its integrated state (prophage) is the reversal of integration and requires recombination of the P and P' sites on the prophage. For efficient excision the products of both the int and xis genes are required. Little is known about the regulatory mechanisms of genes int and xis; it is paradoxical how the phage genome manages to stay integrated when both integration and excision are equally efficient and the int and xis genes belong to the same operon N. It also

remains to be defined which cellular and/or viral factors influence the outcome of an infection of *E. coli* by lambda, as well as what consequences for the host cell result from integration of the viral DNA.

To conclude, it is important to stress, despite the incomplete picture, that a phage encoded, highly efficient recombination system exists leading to the insertion of the phage lambda DNA into the bacterial chromosome at specific sites.

The temperate bacteriophage Mu of *E. coli* K12, is very different from lambda with regard to the molecular events of its life cycle. The genome of the mature Mu bacteriophage is a linear DNA duplex molecule of molecular weight 28×10^6 (Torti *et al.*, 1970; Martuscelli *et al.*, 1971). A feature of Mu DNA is the presence of a 3000 base pair sequence located near the S end which can undergo inversion. The sequence, called the G segment, can invert by recombination between identical, but inverted, sequences of about 50 base pairs flanking the G segment (Hsu and Davidson, 1974). It has been shown that the invertible G segment affects the infectivity of progeny Mu particles (Bukhari and Ambrosio, 1978; Kamp *et al.*, 1978). Only Mu particles containing the G DNA fragment in one orientation (called + or flip) are infectious, while those containing the G DNA fragment in the opposite orientation (- or flop) fail to infect, apparently as a result of the inability of their progeny to absorb to the cells. However, the most striking and unique property of the Mu DNA is the nature of its ends. When the two DNA strands are separated and reannealed, complete duplexes are not regenerated. Instead one end, identified as the right or S end, is always split into two single stranded tails, visible in the electron microscope (Daniell *et al.*, 1973; Hsu and Davidson, 1974). The single stranded tails are variable in length (0.5 - 3 kb) and their failure to renature reflects the heterogeneity of their sequences. Studies on the renaturation kinetics of the Mu DNA have

implied that *E. coli* DNA sequences at the S end are responsible for their heterogeneity (Daniell *et al.*, 1975). It has recently been shown conclusively that *E. coli* DNA is present at the S end and also that a shorter *E. coli* sequence exists at the opposite C (left) end (Bukhari *et al.*, 1976), as had been previously suggested by Allett and Bukhari (1975). The end sequences represent different parts of the *E. coli* chromosome and the amount of host sequence at the C end (70 - 150 base pairs) is about tenfold less than that present at the S end. Mu is so far the only viral system in which mature viral DNA acquires host sequences during normal replication. The temperate bacteriophage genomes generally have either cohesive ends or have directly repeated end sequences which are used to convert the linear DNA molecules into circular forms. Bacteriophage Mu has heterogenous sequences at the ends of its DNA and has no obvious means of fusing them to form circular molecules. The Mu DNA does, however, integrate into the *E. coli* chromosome, in both lysogenic and lytic infection, as I shall discuss later. A striking feature of this phage is that it causes random polar mutations in a large number of the genes of the lysogenised host (Taylor, 1963; Martuscelli *et al.*, 1971; Bukhari and Zipser, 1972). Such mutations have been correlated with the location of the insertion of the phage genome into the host chromosome (Martuscelli *et al.*, 1971; Boram and Abelson, 1971; Bukhari and Zipser, 1972). The Mu genome can be integrated in the *E. coli* chromosome randomly, irrespective of the host sequences encountered (Bukhari and Zipser, 1972). In this respect the temperate *E. coli* phage Mu differs markedly from other temperate phages, like lambda or P22 which have one specific site of integration or phage P2 which has a limited number of attachment sites (Calendar and Lindhal, 1969). There is evidence that the Mu phage genome can be inserted in a clockwise or counter clockwise orientation with respect to the bacterial chromosome (Abelson

et al., 1973), and that the genetic and physical map of prophage and vegetative phage are congruent (Hsu and Davidson, 1972; Abelson *et al.*, 1973). The topography of integrative recombination must therefore differ from that of λ. Little is known however about the enzymatic mechanism that is responsible for the insertion of Mu DNA except that it is phage encoded, recognises a specific site on the phage DNA and has apparently no specific requirements as to recognition of host sequences.

As mentioned previously, random integration of Mu DNA into the host chromosome also occurs during lytic development, and following induction of prophage (Razzaki and Bukhari, 1975). Ljungquist and Bukhari (1977) have shown that the original junctions between prophage and host DNA persist after induction until late in the lytic cycle. It is implied that the prophage DNA remains at its original location when Mu DNA is actively replicating and apparently many copies of Mu DNA have been integrated into the host DNA. It is therefore postulated that the Mu integrative precursor, the form of DNA that is inserted into the host DNA, is generated by replication of Mu DNA.

Finally, the packaging of Mu DNA apparently occurs from maturation precursors that contain Mu DNA covalently linked to host DNA (Bukhari and Taylor, 1975). The host sequences of Mu DNA ends have thus been postulated to arise by headful packaging of such maturation precursors. This would mean that the headful packaging of Mu DNA starts from the C end, linked to host DNA, and proceeds towards the S end, also linked to host DNA, and that the presence of host DNA at the S end is a phenomenon of the size of Mu DNA. The acquisition of 75-150 host base pairs at the C end is apparently a specific event, unrelated to the size of Mu DNA being packaged (Bukhari *et al.*, 1976). Thus, bacteriophage Mu does not conform to the standard mode of integration-excision of temperate bacteriophage. The simple recombinational event proposed by Campbell

(1962) to explain the integration, and by a reverse process the excision of lambda DNA from the host chromosome, does not apply to bacteriophage Mu. Also, Mu DNA extensively re-integrates into the host chromosome during lytic infection, while during lytic infection with bacteriophage lambda the integration functions are repressed (Herskowitz, 1973).

Up to this point, lysogeny has been analysed with respect to the molecular and genetic properties of the infecting bacteriophage, which is the carrier of the genetic information necessary to establish lysogeny. Lysogeny, however, must also be considered in relation to its effect on the infected host.

Lysogenic cells carrying a prophage become immune to lytic superinfection with bacteriophage of the same strain. This is known to occur via repression of the lytic genes in the superinfecting phage by the repressor produced by the prophage in lysogenic cells. An induced prophage can also transduce a restricted group of genes, which are located near the prophage insertion site. The gal region is, for example, transduced by the λ prophage after this has been induced. The bacterial phenotype can also be altered by the presence of certain prophages; this phenomenon is called 'conversion'. The 'converting' genes are, however, also active during lytic infection. One example of phage conversion is the production of diphtheria toxins by *Corynebacterium diphtheriae*. The temperate phage β controls the synthesis of toxin which is produced only as long as lysogeny persists. Another example of lysogenic conversion is the alteration of surface antigens in *Salmonella typhimurium* cells. These new properties conferred on the lysogenised cells by the prophage are inherited and as such they have to be viewed as new genetic characteristics of the lysogenic cell.

The DNAs of lysogenic bacteriophages such as Mu and lambda, may be thought of as very large translocatable elements, as mentioned above. Their insertion into the bacterial chromosome is rec-A independent. As described, Mu DNA can insert at a large number of sites on the host chromosome, while lambda DNA normally integrates at a specific single site. However, when the normal specific single site on the host chromosome is deleted, lambda DNA integrates, at much lower frequencies, into many other sites. Both lambda and Mu cause mutations in the bacterial chromosome by inserting their DNAs within structural genes and cause polar effects on the expression of distal genes when their DNAs are inserted in an operon (Adhya and Shapiro, 1969; Bukhari and Zipser, 1972; Daniell *et al.*, 1972; Shimada *et al.*, 1973; Berg *et al.*, 1975). The same polar effect is exerted also by most of the translocatable elements. Interference by integrated prophage with the expression of neighbouring genes is observed in the increased repression of the gal operon by the adjacent lambda prophage (Adhya *et al.*, 1974). The gal operon can be transcribed not only by the normal gal cellular promoter (P_{gal}), but also from the P_L promoter of bacteriophage lambda, under the control of lambda regulatory functions (Adhya *et al.*, 1974). It has been found that when the gal operon is under phage control, it becomes discoordinately transcribed and expressed (Merrill *et al.*, 1978).

There is evidence that most of the mutations induced by insertion of translocatable elements in a structural gene revert at detectable frequencies (Shimada *et al.*, 1973; Ahmed and Johansen, 1975; Bukhari, 1975; Starlinger and Saedler, 1976). However, a small proportion of such mutations will become stable. Of the mutations caused by insertion of Mu prophage into the lac gene, as many as 25 percent fail to revert. Non-reverting mutants carry a deleted lac gene (Howe and Zipser, 1974; Bukhari, 1975; Cabezon *et al.*, 1975). It is thus legitimate to look at

bacteriophages as possible mutating agents, able to cause stable and inheritable alterations in the lysogenised cells. In this and other respects bacteria lysogenised by bacteriophages resemble cells transformed by animal viruses, which will be discussed below.

ANIMAL VIRUSES

RNA TUMOUR VIRUSES

RNA tumour viruses are widely spread throughout the animal species. Rats, mice, birds, cats, hamsters, monkeys and other animals are hosts to RNA tumour viruses. Most RNA tumour viruses induce sarcomas and leukaemias, which are tumours of the connective tissue and of the haemopoietic-reticuloendothelial system respectively. The only RNA tumour virus which is known to cause carcinomas is mouse mammary tumour virus (MMTV). Carcinomas are malignant tumours of epithelial origin and are the most common tumours in humans. Breast cancer is the human analogue of the mouse mammary carcinoma. Although this has not been unequivocally demonstrated, there is considerable evidence of RNA tumour virus particles in human breast tissue and milk (Moore *et al.*, 1971; Dion and Moore, 1972; Sarkar and Moore, 1972) and of RNA tumour virus components, e.g., RNA and reverse transcriptase, in human leukaemia (Spiegelman *et al.*, 1973; Todaro and Gallo, 1973; Gallo *et al.*, 1973; Mak *et al.*, 1975). No infectious RNA virus particles have ever been isolated from biopsies of human tumours.

The RNA tumour viruses can be classified on the basis of their morphology, as revealed in the electron microscope, into A-type, B-type and C-type particles (Gross, 1970; Sarkar *et al.*, 1972). All mature leukaemia and sarcoma RNA viruses have a C-type morphology, while MMTV has a B-type morphology. The response of a cell to infection with an RNA

tumour virus is complex. RNA tumour virus do not show a clear-cut distinction between productive infection and transformation. Cells infected by these viruses are not killed, and virus replication can be accompanied by cell transformation. Whether infected cells become transformed, or yield virus, or both, depends on many parameters including the genotype and the phenotype of both the cell and the virus. Permissive cells are defined as those which support the replication of the virus and are converted to a transformed state by the tumour virus, whereas non-permissive cells do not support virus growth, but may be transformed at low frequency (Temin, 1971). In general, RNA viruses replicate only in cells of the same species as that of their natural hosts. Sarcoma viruses transform fibroblasts *in vitro* and they can be assayed by focus formation in fibroblast monolayers. Leukaemia viruses do not transform fibroblasts in culture, though they replicate in them. However, they can transform lymphopoietic or haemopoietic cells in culture and induce lympho-proliferative disease in animals.

RNA tumour viruses contain a complex of several single stranded RNA species (Robinson *et al.*, 1965; Robinson and Baluda, 1965; Duesberg and Robinson, 1966; reviews by: Duesberg, 1970; Temin, 1971), which sediments at 60-70 S in neutral sucrose gradients and has a total molecular weight of approximately 10^7. Upon exposure to denaturing conditions, this complex dissociates into a major component with a sedimentation coefficient of about 35 S and a molecular weight of 2.5×10^6 to 3.3×10^6, and several small (4 to 10 S) RNA species (Duesberg, 1968; Erikson, 1969). Electron microscopy of the 60-70 S RNA species suggests that it is composed of two 35 S RNA molecules with identical nucleotide sequences linked together by their ends (Delius *et al.*, 1974; Baluda *et al.*, 1974; Duesberg *et al.*, 1974; Kung *et al.*, 1975).

Terminally repeated sequences have been found at the ends of the 35 S RNA genome of Rous Sarcoma Virus (RSV) and Avian Sarcoma Virus (ASV). An RNA sequence, twenty-one bases long, at the 3' end, next to a poly (A) region, is identical to the sequence found adjacent to the 'cap' at the 5' end (Haseltine *et al.*, 1977; Schwartz *et al.*, 1977; Coffin and Haseltine, 1977; Collett *et al.*, 1977). The 4 S RNA species released upon denaturation of the 60-70 S complex is t-RNA which serves as primer during the transcription of the viral genome (Canaani and Duesberg, 1972; Faras *et al.*, 1974; Haseltine *et al.*, 1976). Associated with the virion of RNA tumour viruses, and encoded in the viral genome (Linial and Mason, 1973; Wyke and Linial, 1973) is the enzyme reverse transcriptase, an RNA-dependent DNA polymerase (Temin and Mizutani, 1970; Baltimore, 1970; Temin and Baltimore, 1972).

The discovery of the reverse transcriptase finally supplied an explanation for numerous experiments suggesting the involvement of a DNA intermediate in the replication of the RNA tumour viruses (Temin, 1963; 1964; Bader, 1964; Bader and Bader, 1970; Balduzzi and Morgan, 1970; Boettiger and Temin, 1970). To account for the unexpected data hinting at the participation of a DNA intermediate in the replication of the viral RNA, Temin proposed, in 1964, his provirus hypothesis. He suggested that a DNA copy of the infecting single stranded RNA, the provirus, is the replicative intermediate of the RNA tumour viruses and becomes integrated into the host cell chromosome. The discovery in 1970 of the enzyme reverse transcriptase in Rous Sarcoma and Rauscher mouse leukaemia virions (Baltimore, 1970; Temin and Mizutani, 1970) satisfied the requirement of the provirus hypothesis, for an enzyme capable of transcribing DNA from viral RNA. Reverse transcriptase is found in all RNA tumour viruses (Temin and Baltimore, 1972). The enzyme promotes synthesis of DNA and accepts natural and synthetic RNA and DNA as template.

Hausen and Stein (1970) demonstrated that there was also a ribonuclease, called H, associated with the polymerase and inseparable from it by physical techniques (Baltimore and Smoler, 1972; Keller and Crouch, 1972). Ribonuclease H specifically degrades the RNA strand of an RNA-DNA hybrid but does not degrade either single or double stranded RNA (Hausen and Stein, 1970). Ribonuclease H may participate in the replication of DNA by removing primers from the template subsequent to the initiation of DNA synthesis (Keller and Crouch, 1972; Berkower *et al.*, 1973). Molecular studies now provide definite evidence for both the existence of a provirus and the central role of the provirus in viral replication and virus induced transformation.

After entry of the virus into the cell, the virion RNA-dependent DNA polymerase becomes activated (Temin and Mizutani, 1970; Baltimore, 1970) and transcribes the high molecular weight virion RNA first into single-stranded and then into double-stranded DNA (Temin and Baltimore, 1972). Viral DNA is synthesised in the cytoplasm in the first few hours after infection (Hatanaka *et al.*, 1971; Varmus *et al.*, 1973). This cytoplasmic viral DNA appears as both linear and circular duplex molecules of a length similar to that of a subunit of the viral RNA genome (Guntaka *et al.*, 1975). Most of these molecules have a covalently continuous full length strand of DNA which is complementary to the viral genome ('minus' strand) and a 'plus' strand which contains a number of nicks, that is, it is not covalently continuous (Gianni and Weinberg, 1975; Varmus *et al.*, 1976). Viral DNA then migrates to the nucleus where it is detected as a covalently closed supercoiled circle just prior to integration into the host chromosome (Gianni *et al.*, 1975; Varmus and Shank, 1976). It has been shown that the linear cytoplasmic DNA is a precursor of the covalently closed circular DNA in the nucleus (Shank and Varmus, 1978). Since the finding that the 35 S RNA contains terminally repeated sequences

at its ends (Coffin and Haseltine, 1977; Collett *et al.*, 1977; Haseltine *et al.*, 1977; Schwartz *et al.*, 1977), possible ways of circularisation of the molecule can be envisaged.

The viral DNA, after entry into the nucleus and circularisation, integrates into the host chromosome. Apparently the supercoiled circular DNA form is a necessary requirement for integration, since ethidium bromide reduces the formation of superhelical turns and concomitantly blocks the integration of the viral genome (Guntaka *et al.*, 1975). It seems also that integration of viral DNA requires cellular DNA replication (Varmus *et al.*, 1977). The first physical evidence for the existence of an integrated provirus, as postulated by Temin, came from the work of Varmus (Varmus *et al.*, 1973). A new technique was developed to test for integration of virus specific DNA into the DNA of higher organisms. This is called the 'network' technique and is based on the presence of extensively repeated sequences in the high molecular weight DNA of eukaryotic cells. When unsheared denatured cell DNA is incubated at Cot values (nucleotide molar concentration x time in seconds) which allow repeated, but not unique, sequences to reanneal, networks of DNA are formed; these can be separated from the rest of the DNA by sedimentation. The finding of virus-specific DNA in networks demonstrates covalent linkage of viral DNA to cellular DNA containing repeated sequences, and thus, its integration in the cell genome.

This approach was used to study RSV-specific DNA in both: (i) duck embryo fibroblasts which are transformed by RSV, and support its replication; and (ii) BALB/c 3T3 mouse cells which are inefficiently transformed, and do not support RSV replication. Cellular DNA networks were prepared from these cells after transformation by RSV, and were assayed for viral sequences by DNA-DNA reannealing kinetics (Gelb *et al.*, 1971).

Different amounts of RSV-specific DNA were detected integrated in the DNA network of transformed duck cells (4-6 viral DNA equivalents per cell) and of transformed mouse cells (0.8 viral DNA equivalents per cell). No viral-specific DNA could be detected in uninfected cells.

The network assay was also used to test for integration of viral DNA in mouse and duck cells 12 h after infection with RSV. Unintegrated as well as integrated sequences were found. This further validated the network method as a test for integration of viral sequences, since it distinguished between free and integrated sequences. The method, as originally described, did not take into account that free viral DNA can contaminate the network pellet of cell DNA after sedimentation.

Permissive and non-permissive cells infected with ASV were also analysed for integrated and non-integrated viral DNA sequences (Varmus *et al.*, 1976). Infected cell DNA was fractionated either by network formation or by sedimentation in alkaline sucrose gradients in a zonal rotor. Integrated and non-integrated viral DNA were detected using either of these procedures. The agreement of the results obtained indicated that both methods are adequate to separate high molecular cell DNA to be tested for integrated viral sequences from free viral DNA. Unintegrated virus-specific DNA consisted of duplexes with 'minus' strands of the length of subunit (35 S) RNA, and relatively short fragmented 'plus' strands (Varmus *et al.*, 1976). This form of the DNA was shown to be confined to the cytoplasm.

Hill and Hillova (1972; 1974) showed that the entire genetic equivalent of RSV required for transformation (and infectivity) was associated with the genome of RSV transformed mammalian cells. Cell DNA from non-permissive XC rat cells transformed by the Prague strain of RSV was purified, after denaturation in alkali and neutralisation, by sedimentation in neutral CsCl gradients. This purified cell DNA was

shown to have the capacity, both in the native and in the denatured form, to transform chicken embryo fibroblast cells. After sedimentation in alkaline buoyant density gradients or in alkaline glycerol gradients, XC cell DNA maintained its transforming capacity. In these gradients the cellular DNA sediments at a different buoyant density or at a different rate from free circular (or linear) viral DNA and is thus very likely to be free of viral contaminants. Therefore, the viral sequences associated with the cellular DNA, and responsible for infectivity, must be integrated.

However, integration of virus-specific DNA is not restricted to infected cells. In early attempts to find evidence to support the provirus hypothesis, Temin (1964) looked for viral DNA sequences in RNA tumour virus infected cells. The finding that virus specific sequences were present in control as well as in infected cells did not then seem to support the provirus hypothesis, and the true significance of the data were not understood. However, since the introduction of more sensitive methods for the detection of viral DNA sequences, further evidence has accumulated for the presence of RNA tumour virus specific DNA sequences in uninfected cells.

Uninfected cells of many species contain the genetic ability to produce C-type viruses. Mice also harbour the genes of B-type viruses (Varmus *et al.*, 1972; Nandi and McGrath, 1973; Lieber *et al.*, 1973). The presence of virus-specific DNA sequences in uninfected cells has been demonstrated by RNA-DNA filter hybridization (Rosenthal *et al.*, 1971; Baluda, 1972) and by reannealing kinetics with labelled virus-specific DNA probes (Varmus *et al.*, 1972; Markham and Baluda, 1973; Varmus *et al.*, 1976). These methods will be described in a later section. Markham and Baluda (1973) showed that virus-specific DNA sequences were associated with high molecular weight DNA fractionated from normal chicken embryo

fibroblasts, and from virus-producing leukaemic cells transformed by AMV, by sedimentation in alkaline sucrose gradients. This suggested that the viral DNA sequences were integrated into the cell DNA.

The expression of endogenous viral genes (i.e., not derived from any exogenous viral infection) in uninfected cells can be partial or complete and is subject to a genetically determined regulation. A significant example of partial expression is the presence of viral specific antigens in otherwise normal cells (Payne and Chubb, 1968; Taylor *et al.*, 1971). In chickens and mice this property segregates like an autosomal trait of the cellular genotype and correlates with the presence of RNA transcribed from endogenous provirus (Payne and Chubb, 1968; Taylor *et al.*, 1971; Hayward and Hanafusa, 1973). These data suggest that the viral DNA is integrated into the cellular genome. Other manifestations of the expression of endogenous viral genes are the presence of virus-specific RNA in apparently uninfected cells lacking the group specific antigen (Hayward and Hanafusa, 1973; Benveniste *et al.*, 1973), and the capacity of some normal cells to complement the replication of exogenous viruses (Weiss and Payne, 1971; Hanafusa *et al.*, 1972). Spontaneous production of endogenous virus has been observed, mostly in chickens, mice and cats (Vogt and Friis, 1971; Todaro, 1972; Lieber *et al.*, 1973; Livingston and Todaro, 1973). Production of endogenous virus can also be induced by treatment of cultured cells with a variety of physical and chemical agents (Weiss *et al.*, 1971; Aaronson *et al.*, 1971).

All these data support the conclusion that most avian and murine cell lines contain the genome of an RNA tumour virus, either in part or complete, and that this genetic information can be variably expressed or activated. They strongly suggest that the virus specific information is transmitted vertically from generation to generation in the form of integrated DNA provirus.

One important aspect of the integration event concerns the sites of integration on the host DNA, the number of viral copies, their specificity, and the arrangement of the integrated DNA sequences. Information about these features of integration could improve our understanding of the mechanisms by which integration occurs and, possibly, the regulation of the expression of integrated viral genes. The localisation of genes in eukaryotic cells is difficult because of genomic complexity and scarcity of characterised cellular markers. However potential markers are represented by the highly reiterated sequences of eukaryotic DNA (Britten and Kohne, 1968), and integrated viral DNA can be localised in relation to them. Denatured DNA sequences reanneal at a rate proportional to their concentration. Therefore cellular DNA fragments containing reiterated sequences will reanneal faster than fragments of the same size containing unique sequences. Cell DNA can thus be fractionated into components with different sequence reiteration on the basis of their difference in rate of reannealing. The amount of DNA reannealed, i.e., double stranded, can be estimated by chromatography on hydroxylapatite and then tested for viral sequences.

Evans *et al.* (1974) reported that in normal chicken cells, endogenous viral DNA, partially homologous to AMV RNA, appeared to be associated with reiterated cell sequences, and that it appeared that viral DNA was integrated in segments with a maximal size approximately similar to that of the 35 S AMV RNA. On the other hand, in leukaemic cells additional AMV sequences appeared to be integrated adjacent to unique cellular DNA, or in tandem with endogenous viral DNA. Variations in the integration site of RNA tumour virus DNA in different hosts were reported by Dastoor *et al.* (1977). It appeared that the proviral DNA of avian RNA tumour viruses could integrate into host DNA at sites which differ in sequence reiteration frequency.

The pattern of integrated avian sarcoma virus DNA sequences was investigated in six ASV transformed mammalian cell lines thought to contain no free viral DNA (Collins and Parsons, 1977). DNA from the ASV transformed cell lines was digested with three restriction endonucleases, fractionated by agarose gel electrophoresis, transferred to nicrocellulose filter strips, and hybridised with ^{32}P-c-DNA probes. Each of the transformed cell lines gave one or more common viral-specific DNA fragments after digestion with the three restriction enzymes suggesting that the same site on the circular provirus may be involved in recombination with the host DNA in all six AMV transformed cell lines examined. These experiments, however, did not unequivocally establish whether integration of the ASV specific DNA was at unique cellular site or at different sites within the host cell DNA.

Khoury and Hanafusa (1976) and Battula and Temin (1977; 1978) have also attempted to test if integration of provirus is limited to specific sites in the cell DNA after RNA tumour virus infection. They measured the variation in the number of copies of virus-specific DNA integrated in chicken embryo fibroblasts after RAV-2 infection with different multiplicities and at short times, long times, and several transfers after infection (Khoury and Hanafusa, 1976). The finding that viral infection caused the permanent addition of a constant number of copies of integrated viral DNA sequences, independent of the multiplicity of infection, is consistent with the hypothesis that chicken cells contain a limited number of specific integration sites for the viral DNA.

After infection by spleen necrosis virus (SNV) and other reticuloendotheliosis viruses, avian cells have a phase of virus production accompanied by cytopathic effect and death of some cells. This phase is called acute infection. After this phase, the cytopathic effect disappears and the cells regain a normal appearance. This stage is

called chronic infection. Battula and Temin (1977) showed that SNV specific DNA was integrated at a unique site in chronically infected cells and at multiple sites in acutely infected chicken cells. The integrated virus specific DNA was infectious, which indicated that the entire viral information was present. It was postulated then (Battula and Temin, 1977) that death of the cells in the acute infection was due to the integration of the infectious viral DNA at multiple sites. This hypothesis was further tested by comparing the patterns of integration of infectious DNA in fibroblasts of different avian species chronically and acutely infected, at various multiplicities, with two avian reticuloendotheliosis viruses (Battula and Temin, 1978). The results demonstrated that infectious DNA was integrated at multiple sites in acutely infected cells and at a single site in chronically infected cells. The pattern of integration was independent of the multiplicity of infection used.

Integration of virus specific DNA into the DNA of cells infected by RNA tumour viruses has thus been demonstrated by both genetic and molecular techniques, but relatively little is known about its role in the virus life cycle. The relationship between integration of viral DNA and virus production has been explored by Guntaka *et al.* (1975).

Integration of virus specific DNA into host DNA was found to be inhibited in ethidium bromide (EtBr) pretreated cells while normal levels of viral DNA were synthesised. It was proposed that EtBr acted by interchelating with double stranded viral DNA thus preventing, or reverting, the formation of supercoiled DNA necessary for integration. The suppression of integration led to a coordinate inhibition of virus production; moreover the effect of EtBr on virus production was appreciable only if the inhibitor was added prior to integration. Since viral RNA and progeny virus were not made when integration of viral DNA

was blocked by EtBr it was concluded that integration is a compulsory step for the full expression of the viral genome. Integrated proviral DNA is also the presumptive template for the synthesis of viral RNA (Temin, 1971). No other template, i.e., complementary RNA, has been found in infected cells (Coffin and Temin, 1972; Bishop *et al.*, 1973) and inhibition of DNA dependent RNA synthesis affects both the production and the synthesis of viral RNA (Temin, 1971). Synthesis and integration of viral DNA has also been shown to be a requirement for virus-induced cell transformation. Ethidium bromide while blocking integration of RSV provirus, prevents cell transformation as well as viral replication (Varmus *et al.*, 1973).

New viral genetic information is added by integration to the DNA of virus transformed cells. These viral sequences will be totally or partially new to the host depending on the pre-existence of endogenous viral sequences homologous to those of the infecting virus. Among these integrated sequences are the ones responsible for transformation. However, viral genes can be integrated into the host DNA without causing transformation of the host cell. This point is illustrated best by the case of avian sarcoma virus infected hamster cells, which reverted from the transformed to the normal phenotype (Macpherson, 1971). The revertant cells retained the viral ASV genome, as shown by rescue of the virus (Boettiger, 1974), which is transcribed, though at lower levels than in the transformed counterparts. However the revertant cells showed no phenotypic evidence of viral gene expression. Similarly normal cells from mice inherit mouse mammary tumour virus, presumably as an integrated provirus (Nandi and McGrath, 1973; Varmus *et al.*, 1972; 1973). It appears then that in the cell some regulatory mechanisms exist which can repress or switch on the expression of viral genes. Even though it is generally assumed that the integrated virus specific DNA is under the same control

of regulation and expression operating on cellular genes, very little evidence for this type of control exists.

DNA TUMOUR VIRUSES

Herpesviruses, Papovaviruses and Adenoviruses represent the three most important groups of DNA tumour viruses. Each of the above groups of DNA viruses provides favourable experimental material for the investigation of a wide range of biological phenomena. They have been shown to cause tumours in animals, to transform cells in culture, and there is a strong possibility that at least some herpesviruses may be related to human cancer. Whatever role the DNA tumour viruses might actively play in the cause of human cancer, however, they offer a suitable model system for studying *in vitro* cell transformation; extrapolation of the results from this system might help in understanding *in vivo* oncogenicity. Papovaviruses and Adenoviruses have been especially useful in the study of DNA structure and replication, offering the advantage of a small genome size and of the availability of large quantities of viral DNA. The small size of their viral genome makes it also likely that a number of cellular functions are involved in the replication of viral DNA. In this regard studies on viral DNA functions and replication could lead to a better understanding of the eukaryotic DNA replication process. With both Papovaviruses and Adenoviruses the outlines of a DNA replication scheme are now available; temperature sensitive mutants, localised on the physical map of the viral chromosome, have made possible the identification of the viral gene products necessary for viral DNA replication. It is interesting that some of these same functions are also necessary for viral transformation of cultured cells. Thus, the investigation of DNA replication, in all its aspects, is useful,

not only because it is an integral part of lytic infection, but also because it is relevant to our understanding of viral transformation and of viral and cellular gene expression. The occurrence of integration of viral DNA into host DNA and its relation to these events will now be discussed for each group of DNA tumour viruses.

Herpesviruses

Herpesviruses have a unique importance among DNA tumour viruses as the most likely viruses to act as etiological agents of natural cancer in animals and in men. Among DNA tumour viruses only they are able to cause tumours in their natural hosts. For obvious reasons research on herpesviruses has been concerned mostly with the various aspects of proven or suspected herpesviral oncogenesis. Investigations on herpesviruses have been focused on five main areas: Marek's disease herpesvirus (MDV) in chickens, Lucké adenocarcinoma in frogs, Epstein-Barr virus (EBV) of man, herpesviruses Saimiri (HVS) and ateles (HVA) of monkeys and viral transformation induced by inactivated human herpes simplex virus (HSV) in various target cells.

While it has been uniquivocally demonstrated that Marek's disease in chickens and Lucké's adenocarcinoma in frogs are caused by herpesviruses, the evidence for a causative association of the other herpesviruses mentioned above with animal or human malignancies is still circumstantial and incomplete. The oncogenic potential of these herpesviruses has been supported by *in vitro* viral transformation experiments.

Herpesviruses can either kill the cells in which they replicate lytically or establish a virus-cell interaction leading to transformation of the target cell. A third possibility *in vivo* is latent infection, the molecular basis of which is not known. It appears that lymphatic cells

are the main target for cell transformation by herpesviruses, although non-lymphatic tissue is also transformed by some agents of this group. However, these *in vitro* transformation experiments while they reinforce the hypothesis that herpesviruses may act as oncogenic agents, by no means prove it.

Unfortunately the molecular mechanism of transformation by herpesviruses is not understood and the molecular biology of the interaction between the host cell and the oncogenic herpesviruses is still at a very early stage. Investigation of the molecular structure of herpesviruses has been restricted mainly to the human HSV and, increasingly, to the simian HSV and HVA. Biochemical and molecular studies with EBV have unfortunately been hindered by the lack of a tissue culture system that produces large amounts of virus. Investigation of the presence of viral DNA sequences in EBV infected cells, either from tumour biopsies or from *in vitro* transformed lines, led to the detection of EBV DNA sequences integrated into the cell DNA. So far, integrated viral DNA sequences have rarely been detected in cells infected or transformed by herpesviruses other than EBV. However, each of the herpesviruses mentioned above will now be individually discussed with respect to the molecular structure of the genome, occurrence of the virus in natural tumours and transforming capacity *in vitro*.

1. Herpesvirus simplex (HSV-1 and HSV-2)

Herpesvirus simplex type 1 and type 2 (HSV-1 and HSV-2) have been intensively studied with regard to their molecular organisation and regulation. They thus represent a useful model for the investigation of the molecular structure of other herpesviruses. Virion HSV DNA is linear and double stranded (Kieff *et al.*, 1971), with an approximate

molecular weight of 100 x 10^6 (Kieff *et al.*, 1971; Frenkel and Roizman, 1972; Wilkie, 1973). When HSV DNA is alkali denatured or released from virions under alkaline conditions, single strands with a molecular weight consistent with intact single strands of DNA are detected together with fragments smaller than unit length (Kieff *et al.*, 1971; Wilkie, 1973). Frenkel and Roizman (1972) reported that the intact single strands derived from only one strand of the parental molecule and that the smaller DNA fragments fell into discrete size classes. However, this was contradicted by other results (Wilkie *et al.*, 1974), suggesting the presence of equimolar amounts of both parental strands and an apparently random collection of DNA fragments as a result of alkali denaturation of virion DNA. The fragments observed after alkali denaturation indicate the presence of single-strand interruptions in the native herpesvirus DNA duplex. The nature and the function of these interruptions are not understood. Their presence, however, does not impair the infectivity of the naked DNA (Graham *et al.*, 1973; Sheldrick *et al.*, 1973; Wilkie *et al.*, 1974).

HSV DNA contains internal inverted duplications of its terminal sequences (Figure 2; Hayward *et al.*, 1975). The HSV DNA molecule consists of two fragments, L and S, both containing unique sequences, flanked by a large inverter terminal redundancy (Figure 2). The end sequences, represented in Figure 2 by the letter a, are a direct terminal DNA repeat (Grafstrom *et al.*, 1974). The direct DNA sequence repeat at the ends of the HSV DNA molecule suggests also a mechanism for circularisation of the molecule. Following a limited exonuclease digestion of the duplex DNA terminal repetitions, complementary single stranded DNA ends could be exposed, which on annealing yield cyclic DNA molecules. Four populations of HSV DNA molecules are found in the virions in approximately equal amounts. These molecules differ in the relative orientations of the L and

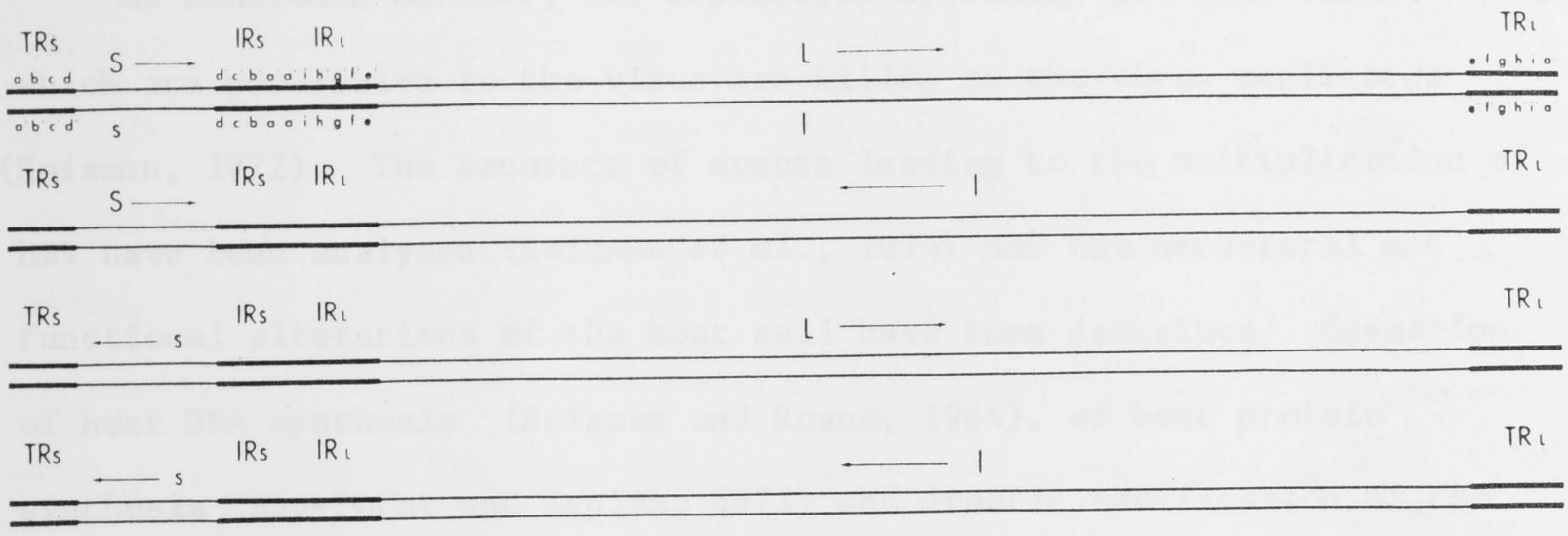
FIGURE 2: Diagram of the structures of herpesvirus DNA (adapted from Subak-Sharpe and Timbury, 1977)

Top: Herpesvirus simplex (HSV-1 and HSV-2) DNA. The four molecules represent the sequentially different molecules that are expected to be generated by intramolecular reciprocal crossing over between the internal repeat and the terminal repeat sequences.

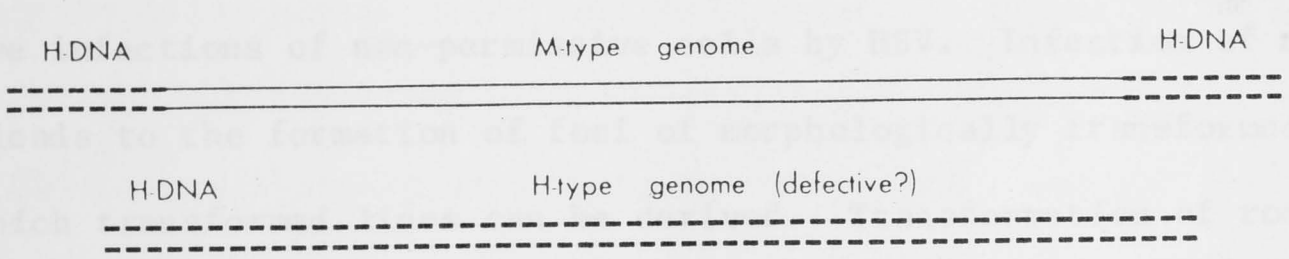
TRs and IRs represent invert repeated sequences flanking the S segment and each accounts for 4.5 percent of the DNA molecule. TR_L and IR_L similarly represent inverted repeated sequences flanking the L segment, and each accounts for 6 percent of the DNA molecule. The S and the L segments contain 9 percent and 70 percent respectively of the viral DNA. TR_L and TR_S sequences differ, but share a common sequence at their respective ends (indicated by a).

Bottom: Simian Herpesvirus (HVA + HVS) DNA (from Fleckenstein *et al.*, 1978). Scheme of the prodominant types of DNA molecules isolated from HVA and HVS particles. H-DNA, represented by a broken line, consists of repeated DNA sequences of high G+C content. L-DNA, represented by a continuous line, consists of unique sequences of lower G+C content.

Herpes Simplex Virus DNA



Herpes Simian Virus DNA (HVA and HVS)



	H-DNA %G+C		L-DNA %G+C
HVA	75	HVA	38
HVS	70	HVS	35.8

S regions (Figure 2; Sheldrick and Berthelot, 1974; Hayward *et al.*, 1975). Such molecules have been suggested to arise either from an intramolecular reciprocal recombination event between the homologous sequences, or by reassortment of physically separated L and S segments.

As mentioned earlier, HSV replicates lytically in human cells. Cells which are permissive to the virus are killed as the virus replicates (Roizman, 1972). The sequence of events leading to the multiplication of HSV have been analysed (Roizman *et al.*, 1974) and the structural and functional alterations of the host cell have been described. Cessation of host DNA synthesis (Roizman and Roane, 1964), of host protein synthesis (Ben-Porat and Kaplan, 1971) and drastic modification of the host RNA metabolism (Roizman *et al.*, 1970) are induced early after HSV infection. Virus specific changes are also induced in the host cell membranes (Roizman and Spear, 1971).

Comparatively little is known about the molecular biology of abortive infections of non-permissive cells by HSV. Infection of rodent cells leads to the formation of foci of morphologically transformed cells from which transformed lines can be derived. Transformation of rodent cells is achieved by infection with partially inactivated HSV. Ultra-violet (U.V.) irradiated wild type HSV-1 and HSV-2 can induce cell transformation as can ts-mutants of both viruses at the non-permissive temperature (Duff and Rapp, 1971; 1973; Macnub, 1974; Rapp and Li, 1974; Subak-Sharpe *et al.*, 1974; Munk and Derai, 1975). Inoculation of these *in vitro* transformed cells into susceptible animals leads, in some cases, to metastasising tumours (Duff and Rapp, 1973; Macnub, 1974; Rapp and Li, 1974; Munk and Derai, 1975). Transformation of rodent cells has also been achieved using sheared HSV DNA from both wild type and ts-mutant HSV (Wilkie *et al.*, 1974). HSV-specific surface and cytoplasmic antigens have been detected in the transformed cell lines, and neutralising antibodies

against the respective transforming agent are found in tumour bearing animals. However, it has been impossible to date to consistently show the presence of HSV DNA sequences in *in vitro* transformed cells and in tumours induced in animals by inoculation of HSV transformed cells. Failure to detect HSV-DNA sequences in the above cells using very sensitive probes (capable of detecting 0.1 HSV genome equivalent per cell; Summers *et al.*, 1975) leads to the conclusion that only small fragments of herpes simplex viral DNA may persist within the HSV transformed cells. At least sufficient viral genetic information remains associated with the host genome to maintain the production of HSV specific antigens. Since transformation can be accomplished with fragmented HSV DNA and conditional lethal mutants, it seems likely that the whole genome of herpes simplex virus is not needed to achieve successful transformation. Results compatible with this hypothesis have been reported by Kraiselburd and Weissbach (1975). Mouse L cells, which are thymidine kinase negative (LTK-) acquire a thymidine kinase (TK) activity after infection with irradiated HSV (Munyon *et al.*, 1971; 1972). This TK activity appears to be virus specific, and the L cells which have acquired it are defined as 'biochemically transformed' by HSV. HSV DNA sequences have been detected in one of these 'biochemically transformed' lines (Kraiselburd and Weissbach, 1975) and multiple copies of a fragment, representing up to 15 percent of HSV DNA are present in the cells.

These *in vitro* transformation experiments with HSV assume a particular importance in the light of the controversy over the role played by herpesviruses in human cancer. HSV-2 has been suggested as a causative agent of human cervical carcinoma (Rawls *et al.*, 1968; Nahmias *et al.*, 1970; Rawls *et al.*, 1973; Rotkin, 1973). The evidence for this association is based on sero-epidemiological data, which are not conclusive at present.

Biopsies from cervical carcinoma tumours have often been tested for the presence of HSV-2 particles and HSV-DNA. Occasionally, HSV-2 virions have been detected (Aurelian *et al.*, 1971) but this represents by itself only circumstantial evidence for a relationship between HSV-2 and cervical carcinoma. Only in one case (Frenkel *et al.*, 1972) has the presence of a fragment of HSV-2 DNA been demonstrated in a cervical carcinoma, as well as its partial transcription into viral m-RNA.

Thus, the role of HSV-2 in cervical cancer is still questionable. The occurrence of cervical tumours in serologically negative patients and the failure to detect viral DNA sequences in the majority of the tumours tested, neither exclude, nor support the suggested causative role of HSV-2 in human tumours. On the other hand, viral DNA sequences have not been consistently detected in *in vitro* HSV transformed cells either, and it might be equally difficult to detect viral DNA segments in *in vivo* tumours. The *in vitro* transforming capacity of HSV boosts the possible role of HSV-2 in human cancer but does not prove it. The relationship between HSV-2 and cervical carcinoma could perhaps find substantial support if an animal system were found in which tumours are induced by virus inoculation.

2. Epstein-Barr virus

Another human herpesvirus which is highly debated as a possible etiological agent of human cancer, is Epstein-Barr virus (EBV). Since the discovery of EBV in lymphoblastoid tissue culture cells from Burkitt's lymphoma by Epstein and his colleagues (Epstein *et al.*, 1964), there has been mounting evidence that this virus is very closely linked and possibly etiologically related to two human tumours: Burkitt's lymphoma (BL) and nasapharyngeal carcinoma (NPC). However, BL and NPC show a peculiar pattern of geographical clustering. BL is endemic in some regions of

Africa, while NPC represents the most frequent malignancy among Cantonese Chinese. EBV infects a majority of the human populations in all countries. EBV primary infection in adults usually causes the benign condition infectious mononucleosis (Henle *et al.*, 1968; Chang and Golden, 1971; Gerber *et al.*, 1972). Infectious (i.e., transforming) EBV is present in the saliva of infectious mononucleosis patients, which also suggests horizontal transmission of the virus by contact. EBV infects almost exclusively B lymphocytes. The remarkable exception to this is the presence of EBV in the epithelian cells of nasopharyngeal carcinomas. Infection of lymphocytes with EBV is not lytic, but abortive in most of the infected cells. It is unfortunate that no tissue culture system in which EBV grows lytically has yet been found. This has made it impossible to obtain sufficiently large preparations of virus for extensive molecular investigations. Very small amounts of EBV virus are obtained from virus-producing cell lines, e.g., P3HR-1 and B95-8. These cell lines have been derived from explantation of a biopsy of a Burkitt's lymphoma, and from *in vitro* lymphocyte transformation with EBV, respectively. In producer cell lines, only a small fraction of the cells (a maximum of 5-10 percent in the P3HR-1 line) produce EBV (Hinuma *et al.*, 1967). The DNA of EBV recovered from the two 'virus-producing' cell lines, P3HR-1 and B95-8, has been analysed (Pritchett *et al.*, 1975). It was found to consist of a linear duplex DNA molecule with a molecular weight of about 100×10^6. However, the EBV DNA from the B95-8 cell line appeared to lack about 15 percent of the sequences of the EBV DNA from the HR-1 line, but all the sequences of the former DNA were presented in the latter DNA. It was suggested that the EBV DNA from the B95-8 line was a deletion derivative of a parental EBV DNA similar in genetic complexity to the EBV DNA from the P3HR-1 line. Possibly, some of the sequences

in B95-8 EBV DNA might have been reiterated to replace the missing region of the putative parental EBV DNA molecule.

As stated above, B lymphocytes are the target cells of EBV infection. Normal lymphocytes have a limited life span in culture. After infection with EBV, however, they are converted into permanently growing cell lines, which are often referred to as being 'immortalised' or transformed by EBV (Pope *et al.*, 1969; Gerber *et al.*, 1969; Miller *et al.*, 1971; Falk *et al.*, 1974). Similar cell lines can be established from the peripheral blood or the lymph nodes of EBV-seropositive donors, either healthy or with benign conditions like infectious mononucleosis (Diehl *et al.*, 1968; Nilsson *et al.*, 1971). The cell lines established from non-malignant tissues are referred to as 'lymphoblastoid' lines as opposed to the 'lymphoma' lines derived from Burkitt's lymphoma biopsies. These cell lines carry multiple copies of the EBV genome, are EBNA (Epstein-Barr virus nuclear antigen) positive and produce virus within a small percentage of the cells (Miller and Lipman, 1973). EBNA is apparently the only virus specific antigen expressed in approximately 100 percent of the cells which harbor EBV genetic information (Reedman and Klein, 1973).

The physical state of the intracellular EBV DNA has been investigated in at least four lymphoblastoid cell lines derived from leukocytes of individuals without lymphoproliferative disease (Kaschka-Dierich *et al.*, 1977). All lines contained multiple copies of viral DNA (from 19 to 100 genome equivalents per cell). Centrifugation in neutral CsCl and CsCl-ethidium bromide density gradients as well as in glycerol gradients was used to separate EBV DNA from cellular DNA. Free circular viral DNA molecules were detected as well as viral DNA with the properties of integrated DNA. As control, two other cell lines derived from tumour biopsies and previously characterised in relation to their intracellular

EBV DNA content, were processed in the same way with reproducible results. They also contained both free circular and integrated EBV DNA (Lindhal *et al.*, 1976). The free circular EBV DNA appeared to be of the same size as that found in EBV virions in all cell lines including the controls, or, at least, the techniques used did not allow any differentiation. Different results were obtained by Adams *et al.* (1977). Circular EBV DNA 12 percent shorter than linear virion DNA was detected as well as integrated DNA sequences, in the 883L lymphoblastoid cell line derived from infectious mononucleosis cells. In addition, three cell lines derived from blood cord lymphocytes immortalised by EBV (produced by the same 883L line) did not contain detectable integrated sequences, but only circular, free EBV molecules of reduced size.

A particular case of cell transformation induced by EBV is that described by Andersson-Anvret and Lindahl (1978). Cell lines, apparently negative for the presence of EBV DNA and EBNA, can be established from undifferentiated malignant B type human lymphomas. Two of these cell lines, the BJAB and the Ramos, can be stably 'converted' *in vitro* by EBV to EBV DNA and EBNA positive sublines (Clements *et al.*, 1975; Fresen and zur Hausen, 1976). The AW-Ramos subline has been shown to contain about one EBV DNA equivalent per cell (Andersson and Lindahl, 1976). Investigation on the intracellular state of this viral DNA has produced results consistent with the presence of viral DNA sequences exclusively integrated in the cellular DNA (Andersson-Anvret and Lindahl, 1978). The viral sequences were exclusively and consistently associated with cellular DNA which had been purified by three different methods: neutral CsCl gradient centrifugation, actinomycin D-CsCl gradient centrifugation and Hirt fractionation (Hirt, 1967). The AW-Ramos, and other analogous cell lines are regarded as 'converted' by EBV, and not as transformed, strictly speaking, since they derive from parent EBV-negative lymphoma cells already transformed by some unknown event.

The tumourigenic potential of the lymphoblastoid cell lines has been judged by their capacity to form colonies in agar (Nilsson and Ponten, 1975) and to induce tumours when transplanted in immunosuppressed or newborn hamsters, rats or mice (Adams *et al.*, 1967; Southam *et al.*, 1969; Adams *et al.*, 1970; 1973). Lymphoblastoid cell lines with a diploid karyotype, which is very common among these lines, neither induce tumours in animals, nor form colonies in agar, but aneuploid lymphoblastoid lines do (Nilsson *et al.*, 1977). The finding that aneuploid lymphoblastoid cell lines can become tumourigenic, as judged by their growth in agar and the induction of tumours in animals, shows that they may acquire some malignant potential *in vitro*, which is expressed, e.g., by a karyotype change and morphological alterations (Nilsson *et al.*, 1974; Nilsson and Ponten, 1975). Thus it might be hypothesised that full development of tumours *in vivo* might require specific genetic changes. The data on *in vitro* transformation of lymphocytes by EBV are strongly supported by the induction of lymphoproliferative disease following injection of EBV into primates (Shope *et al.*, 1973; Epstein *et al.*, 1973; Werner *et al.*, 1975). The induced lymphoma-like tumours contained EBV antigens as well as EBV specific DNA.

As mentioned earlier, EBV has been associated with the human African Burkitt's lymphoma (BL) and Nasopharyngeal carcinoma (NPC). EBV-related antigens as well as EBV DNA have been consistently detected in BL and NPC tumour cells (zur Hausen *et al.*, 1970; Reedman and Klein, 1973; Nonoyama *et al.*, 1973; Lindahl *et al.*, 1974; Kaschka-Dierich *et al.*, 1976). Virus particles have never been detected in tumour biopsies from either tumour. Virus production starts only after explantation of BL cells in tissue culture. Neither virus particles, nor virus antigens associated with virus production, have ever been detected in cultured epithelial cells from NPC. It has not yet been possible to maintain

epithelian cells in long-term tissue culture. In the case of BL derived cell lines, culturing appears to release a block preventing the *in vivo* expression of latent viral DNA. Virus production, however, occurs only in a small percentage of the cultured cells, as exemplified by the P3HR-1 line, mentioned earlier (Hinuma *et al.*, 1967).

Multiple copies of EBV DNA have been detected both in tumour biopsies and in cell lines derived from them, and in both cases the intracellular forms of the DNA have been characterised (Adams *et al.*, 1973; Adams and Lindahl, 1975; Kaschka-Dierich *et al.*, 1976). Tumour biopsies from BL and NPC contain covalently closed and open circular EBV DNA molecules together with some possibly integrated viral DNA sequences (Kaschka-Dierich *et al.*, 1976). The circular EBV DNA molecules have the same size as that of intact DNA from EBV virions. Integrated viral DNA sequences were bound to high molecular weight cellular DNA which had been repeatedly fractionated by neutral CsCl or glycerol gradients. It was perhaps not correct to define such association of viral and cell DNA as integration, since it was not proved to be alkali stable. These results were however consistent with others previously reported (Adams *et al.*, 1973; Adams and Lindahl, 1975). One cell line, Raji, established from a Burkitt's lymphoma, does not produce virus and had earlier been investigated in relation to the intracellular forms of EBV DNA. As in the *in vivo* tumour cells the cultured cells contained integrated and free viral DNA sequences. It was demonstrated that sedimentation in alkaline CsCl gradients of high molecular weight cell DNA previously purified in neutral CsCl gradients, did not affect the association of viral and cellular DNA sequences. The free EBV DNA molecules had sedimentation properties corresponding to those of nicked, circular, full size EBV DNA.

The EBV carrying cell lines derived from Burkitt's lymphomas (or lymphoid lines) differ from the EBV-carrying lymphoblastoid cell lines previously discussed with regard to morphological, functional, and growth characteristics (Nilsson and Ponten, 1975). The most important difference between them is represented by their tumourigenic potential, again judged by their capacity to form colonies in agar *in vitro* (Nilsson and Ponten, 1975) and to induce tumours when transplanted in suitable animals (Adams *et al.*, 1967; 1970; 1973; Southam *et al.*, 1969). As previously discussed, most of the EBV-carrying lymphoblastoid cell lines had a normal diploid karyotype and these did not appear to be tumourigenic. In contrast all lymphoma lines so far examined have aneuploid karyotype, carry various chromosomal abnormalities and are invariably tumourigenic in the way defined above (Manolov and Manolova, 1972; Jarvis *et al.*, 1974; Nilsson and Ponten, 1975). Thus, definite chromosomal differences exist between normal and lymphoma-derived lines. EBV is capable of 'immortalising' B lymphocytes with normal karyotype *in vitro*, but EBV carrying lymphomas *in vivo* involve also genetic changes.

It would be beyond the scope of this review to debate in detail the evidence for the involvement of EBV in BL and NPC. Very generally, it could be summarised that besides the presence of high antibody titers against EBV in BL and NPC patients, and the consistent association of virus specific EBV DNA and proteins with tumour cells, transformation *in vitro* and tumour induction *in vivo* are the strongest arguments for a specific association of EBV with human tumours. Other aspects of this association, like the geographical clustering of BL and NPC tumours, are still unexplained. It has been suggested that the intervention of an environmental or a genetic factor might explain the restricted geographical distribution of NPC and BL.

The molecular data about EBV DNA and its intracellular forms in EBV infected cells deserve further comment. As described earlier, free and/or integrated EBV DNA are found in EBV transformed cells. The free EBV DNA molecules found in BL, NPC and lymphocyte cell lines are circular and full size, that is, of the same size as that of virion DNA. The only exception is the circular EBV DNA of reduced size found in one human lymphoblastic line of infectious mononucleosis origin (Adams *et al.*, 1977).

Unfortunately, no further characterisation of this intracellular EBV DNA has been done; it is not known what sequence homology exists between the different forms of DNA and whether they are infectious and/or transforming.

Integrated EBV DNA has been detected either as the only form of EBV DNA present (Andersson-Anvret and Lindahl, 1978) or together with circular EBV DNA (Adams *et al.*, 1973; Adams and Lindahl, 1975; Lindahl *et al.*, 1976; Kaschka-Dierich *et al.*, 1976). It is not known what relation in size, DNA sequence homology, and parental origin exists between integrated and free circular EBV DNA. It is also not known whether the two forms of EBV DNA are present concomitantly in the same cell. The occurrence of free, circular, and integrated DNA molecules both in tumour biopsies and in established cell lines shows that none of these forms of EBV DNA is a secondary consequence of *in vitro* culturing. The presence of circular DNA molecules might suggest that EBV is carried as an episome during latency, as occurs with extrachromosomal DNA in bacteria. It is known that the temperate bacteriophage P1 replicates its DNA synchronously with the bacterial DNA during lysogeny as a non-integrated circular molecule. P1 DNA can also be present as a defective derivative in an integrated form (Ikeda and Tomizawa, 1968; Scott, 1970). Furthermore, lambda phages mutated in the N gene do not

kill their host bacteria, but persist in them as plasmids (Lieb, 1970). However legitimate this comparison with bacterial systems might be, it shows that the presence of integrated and/or free circular forms of the same DNA molecules have been observed with other viruses. The meaning of the integration event during EBV infection is unknown. The integration of EBV DNA into the DNA of human lymphocytes *per se* does not seem to be sufficient to induce malignancy. It has already been discussed that lymphoblastoid cell lines of the diploid karyotype did not induce tumours in suitable animals (Nilsson *et al.*, 1977; Kaschka-Dierich *et al.*, 1978) though they contained integrated EBV DNA sequences. The hypothesis that the presence of either non-integrated EBV DNA molecules or integrated EBV DNA sequences would be a distinct feature of tumour cells is thus invalid.

To conclude, knowledge of the molecular biology of EBV is still very fragmented and unclear. It has not yet been possible to find a parameter in the EBV system which is consistently correlated with tumourigenicity nor have the molecular events leading to cell transformation or virus replication been characterised. It is thus impossible to assess the significance of the integration event in the EBV infectious cycle or in EBV-induced malignancy.

3. Simian Herpesviruses

Two indigenous primate herpesviruses, herpesvirus saimiri (HVS) and herpesvirus ateles (HVA) have also been increasingly investigated since the discovery of their oncogenic potential (Melendez *et al.*, 1969; Hunt *et al.*, 1970; Melendez *et al.*, 1972). Both HVS and HVA grow lytically in primate kidney cells, and can thus be produced in large quantities. The availability of this permissive system might be of special value for studying the herpesvirus-cell relationship at the

molecular level. The structures of the genomes of HVS and HVA have been characterised. The DNA molecules of these two viruses share two features: a high heterogeneity in base composition and highly repetitive sequences (Fleckenstein *et al.*, 1975; Fleckenstein *et al.*, 1978). At least two types of DNA molecules, denominated M and H, are encapsidated in HVS and HVA virions. M-type genomes contain 'light' unique DNA sequences and highly repeated 'heavy' DNA sequences, while H-type genomes consist exclusively of highly repeated 'heavy' DNA sequences (Figure 2). Nothing is known about the biological function of the described features of these DNAs. It has been found, however, that H-type genomes are not infectious (Fleckenstein *et al.*, 1975).

The oncogenic potential of HVA and HVS has been demonstrated both *in vitro* and *in vivo*. HVA transforms marmoset spleen or blood lymphocytes *in vitro* into continuous lymphoblastic cell lines (Falk *et al.*, 1974; 1978). Transformation is achieved with a fully functional HVA genome, as with EBV. HVA genome expression in transformed cultures is demonstrated by recovery of small amounts of HVA from culture fluids, ability of lymphoblasts to induce infectious centres after co-cultivation with permissive cells, presence of virus specific antigens in the cells. Most of the HVA transformed cells possess T-lymphocyte properties, whereas EBV shows B-lymphocyte tropism. While HVA and HVS cause no apparent disease in their natural hosts, they induce lethal malignant lymphomas when inoculated into various species of primates other than their natural hosts (Hunt *et al.*, 1970; Melendez *et al.*, 1969; 1972). No viral particles are detected in tumour biopsies. However viral antigens and viral particles are induced in lymphoid cell lines established from tumour cells as in the BL derived cell lines. Only a small percentage of the cultured cells produce viral particles. The many

parallels between the mode of infection by simian herpesviruses and EBV have stimulated a good deal of interest in the infection by HVS and HVA. Since there is no lytic system for EBV, the oncogenic primate herpesviruses might offer a promising model system for a better insight in the human cell EBV relationship.

4. Marek's disease and the Lucké adenocarcinoma

A review of the oncogenic herpesviruses would not be complete without a mention of the herpesviruses of Marek's disease in chickens and of Lucké adenocarcinoma in frogs. A herpesvirus in the causative agent of Marek's disease (Witter *et al.*, 1969), a highly infectious lymphoproliferative disease in chickens. The etiology of this disease was definitively proved when vaccination of chickens with attenuated Marek's disease virus (MDV) prevented neoplasia (Churchill *et al.*, 1969). No virus particles are found in the affected lymphatic cells, but viral DNA is present in them in multiple copies (Nazerian *et al.*, 1973). Large amounts of infectious virus are produced within the cells of the chicken's feather follicles (Calnek *et al.*, 1970), and seem to be responsible for the horizontal spreading of the disease. Continuous lymphoid cell lines with characteristics of T-type lymphocytes, have been established from Marek's disease lymphomas (Akiyama *et al.*, 1973; Akiyama and Kato, 1974; Powell *et al.*, 1974). The presence of MDV antigen, MDV capsids and MDV infectious virus have been shown in those cell lines during long term cultivation. While a very small proportion of the cells from these lymphoid lines produces MDV, a large proportion of them harbours MDV DNA (Kato and Akiyama, 1975). One cell line harbours 60-90 MDV genome equivalents per diploid cell (Nazerian and Lee, 1974). Inoculation of cells derived from MDV lymphomas into chickens induces the neoplastic Marek's disease (Kato and Akiyama, 1975), i.e., the MDV associated with these cells is as oncogenic as the original MDV strains.

Another herpesvirus has been shown to be the causative agent of the Lucké tumour (Lucké, 1934), a frog kidney adenocarcinoma. Tumours have been induced upon inoculation into tadpoles of purified herpesvirus from frog tumours (Mizell *et al.*, 1969). Production of viral particles in Lucké tumours depends on climatic conditions. They are synthesised only at temperatures below 12°C and they are practically absent in tumours developed in summer. Lucké virus can, however, be induced to replicate in 'summer' tumours when incubated for prolonged times at low temperature (Rafferty, 1965). The fact that the virus expression occurred in explants from 'summer' tumours (Breidenbach *et al.*, 1971) independently of the intact host, suggested that a complete viral genome was present and latent in the tumour cell.

Papovaviruses

The papovaviruses are divided into two groups: the papilloma viruses and the polyoma-SV40 group (Melnick, 1962). All the papovaviruses have similar chemical composition and structure, containing only protein and DNA. The DNA genome is enclosed in a protein capsid with icosahedral symmetry (Klug and Finch, 1965; Finch and Klug, 1965; Klug, 1965). The papilloma viruses are virtually unstudied with respect to their molecular biology, the main reason for this being the lack of an adequate tissue culture system. However, the polyoma-SV40 group has been thoroughly investigated. These viruses grow to high titers in either primary or secondary cultures of cells derived from their natural hosts, which are mouse for polyoma and monkey for SV40. These cells are thus permissive to virus growth, support the full expression and replication of the viral genome, and are killed following the release of progeny virus.

By contrast, cells from some unnatural host species, e.g., rat cells, are non-permissive for polyoma and SV40 viruses, do not support virus growth, and are not lysed. Virus infection of these types of cells is abortive. Virus transformed cells can arise from an abortive infection of non-permissive cells by SV40 or polyoma virus. Transformed cells are defined in the simplest way as cells which have been altered as a result of viral infection, in their growth pattern, and in the properties of their surface (Tooze, 1973).

Polyoma and SV40 cause tumours in susceptible animals other than their natural host. Similarly, injection of *in vitro* virus transformed cells can cause tumours in appropriate animals. All the biochemical and genetic studies with polyoma and SV40 viruses indicate that they are essentially similar in their viral functions and in the replication of their genome. Information and discussion on the two viruses will therefore be combined.

The virion DNA of the papovaviruses, including the papilloma viruses, is a covalently closed, circular duplex DNA molecule. Its molecular weight ranges from 3.0 - 3.6 x 10^6 in SV40 (Crawford and Black, 1964; Tai *et al.*, 1972; Gerry *et al.*, 1973; Fiers *et al.*, 1978) and polyoma virus (Weil and Vinograd, 1963; Crawford, 1964; Caro, 1965) to 4 - 5.3 x 10^6 in papilloma viruses (Crawford, 1969). The DNA extracted from polyoma and SV40 viruses contains two and sometimes three discrete species of DNA that have been separated and identified by sedimentation in sucrose or CsCl gradients (Watson and Littlefield, 1960; Weil and Vinograd, 1963; Crawford, 1963; 1964; Vinograd *et al.*, 1965). These three forms of DNA have been named component (or form) I, component II, and component III. Component I is a double stranded, covalently closed, DNA helix twisted to form negative superhelical turns (Vinograd *et al.*, 1965; Upholt *et al.*, 1971; Pulleyblank and Morgan, 1975). The presence

of superhelical turns in form I DNA gives the molecule a compact shape which explains its higher sedimentation coefficient, its lower viscosity and its increased resistance to hydrodynamic shearing as compared to the other forms of the DNA molecule. Component II of SV40 and polyoma virion DNA is a double stranded circular molecule containing one or more single-strand interruption(s) in the phosphodiester backbone, allowing free rotation of the DNA strand opposite the polynucleotide chain break (Vinograd and Lebowitz, 1966). Form III DNA is a linear duplex molecule of cellular origin which is packed into an SV40 virus particle. This sort of virus particle, containing host rather than viral DNA, is called a 'pseudovirion'. Papilloma virion DNA also contains three species of DNA equivalent to the components I, II, and III described for SV40 and polyoma viruses (Vinograd *et al.*, 1965; Crawford, 1969).

Within virions or within infected cells, SV40 and polyoma DNA is associated with histone proteins in a structure similar to that of eukaryotic chromatin (Huang *et al.*, 1972; Lake *et al.*, 1973; Griffith, 1975; Germond *et al.*, 1975). The SV40 DNA and the associated histones can be isolated as a nucleoprotein complex from SV40 virions treated with alkaline buffers at pH 10.5 (Huang *et al.*, 1972; Lake *et al.*, 1973). When SV40 DNA is extracted free of proteins from infected cells, different types of molecules are detected: form I, form II, replicative intermediates and oligomers. Oligomeric forms of SV40 DNA up to hexamers can be isolated from infected cells. Both interlocked circular and linear oligomers have been detected (Jaenish and Levine, 1971; Martin *et al.*, 1976). Oligomeric forms represent only a small fraction of the total intracellular SV40 DNA.

Physical maps of the genome of SV40 and polyoma viruses have been obtained by cleavage with a large number of restriction endonucleases (Danna and Nathans, 1972; Sack *et al.*, 1973; Subramanian *et al.*, 1974;

Lebowitz *et al.*, 1974; Griffin *et al.*, 1974). The complete nucleotide sequence of SV40 DNA, which is undoubtedly the ultimate physical map, has also been determined very recently (Fiers *et al.*, 1978). EcoRI restriction enzyme cuts SV40 and polyoma virus DNA at a unique site (Mulder and Delius, 1972; Morrow and Berg, 1972). The DNA map is by convention divided into 100 units beginning at the unique site (0 point) of cleavage by EcoRI and increasing in a clockwise direction. The origin of DNA replication has been localised on the DNA physical maps of SV40 and polyoma viruses around position 0.67 and 0.7 respectively.

With respect to transcription and function, the circular DNA molecules of SV40 and polyoma are roughly divided into two half circles. The half circle from position around 0.67 (SV40) or 0.73 (polyoma) is transcribed into two continuous m-RNA molecules related by splicing and sedimenting at 19-20S (Weinberg and Newbold, 1974; Kamen and Shure, 1976; Berk and Sharp, 1978). Transcription of this region of the DNA is counter-clockwise on SV40 DNA and clockwise on polyoma DNA, and occurs 'early' in infection, i.e., before the onset of viral DNA replication during lytic infection. The 'early' region of polyoma and SV40 DNA codes for at least two proteins: large T antigen of molecular weight 90,000 - 100,000 and small t antigen of molecular weight 15,000 - 22,000 (Tegtmeyer, 1974; Paulin and Cuzin, 1975; Rundell *et al.*, 1977; Staneloni *et al.*, 1977; Fluck *et al.*, 1977). T and t are encoded by the two early m-RNAs related by differential RNA splicing as described above (Berk and Sharp, 1978). Deletions in the DNA sequences specifying the 3' portion of the early viral m-RNA appear to affect the expression of large T whereas deletions in the DNA sequences specifying a portion of the 5' end of the early viral m-RNA appear to affect the small t (Ito *et al.*, 1977; Prives *et al.*, 1977; Crawford *et al.*, 1978; Kamen, personal communication).

The other half of the viral DNA molecule is transcribed from position 0.67 to 0.17 in a clockwise direction on SV40 DNA and anti-clockwise from 0.73 to 0.26 on polyoma DNA, into two partially overlapping m-RNA molecules sedimenting at 16S and 19S (Weinberg *et al.*, 1974; Buetti, 1974). Transcription of this region of the viral DNA occurs 'late' in infection, after the onset of viral DNA replication. The 'late' region of the viral DNA codes for the virus structural proteins. The 16S m-RNA codes for the major virion capsid protein VP1 (Prives *et al.*, 1974; Smith *et al.*, 1975) while the 19S m-RNA codes for two minor capsid proteins VP2 and VP3 (Tegtmeyer, 1974; Smith *et al.*, 1975).

Several temperature sensitive mutants of polyoma and SV40 viruses have been characterised (Eckhart, 1969; Di Mayorca *et al.*, 1969; Tegtmeyer and Ozer, 1971; Kimura and Dulbecco, 1972; Chou and Martin, 1974). They fall into four to five complementation groups: A, B, C, BC and D (Tegtmeyer and Ozer, 1971; Chou and Martin, 1974), and corresponding DNA mutations have been localised on the DNA physical map (Lai and Nathans, 1975; Shenk *et al.*, 1975). So far, among the temperature sensitive mutants, only group A represents an early gene (A) function which maps toward the 3' end, or distal portion, of the early m-RNA (Miller and Fried, 1976; Feunteun *et al.*, 1976). The A gene product is required for initiation, but not for elongation or termination of viral DNA synthesis (Tegtmeyer, 1972; Francke and Eckhart, 1973; Chou *et al.*, 1974). The A gene product appears also to be responsible for the stimulation of cellular DNA replication, which occurs early in the infection of permissive and non-permissive cells with polyoma or SV40 viruses (Tegtmeyer, 1972; Chou and Martin, 1974; Tjian *et al.*, 1978). Finally, the most interesting property of the tsA mutants is represented by their inability to transform cells at the restrictive temperature associated, at least in the case of cells selected in liquid medium, with a temperature dependent

phenotype of cells transformed by tsA mutants at the permissive temperature (Tegtmeyer, 1972; Martin and Chou, 1975; Tegtmeyer, 1975; Brugge and Butel, 1975).

Several experiments have indicated that SV40 and polyoma mutations of the tsA group affect the synthesis and the physical properties of the T antigen (Tegtmeyer, 1974). More recently it has been shown that T antigen is encoded in the sequence of the viral DNA mapping toward the 3' end of the early RNA region (Prives *et al.*, 1977; Paucha *et al.*, 1977; Ito *et al.*, 1977). This is the same region of the early viral DNA in which the A gene maps. It is thus likely that T antigen is the product of the A gene.

The host-range mutants of polyoma virus isolated by Benjamin (1970) also map within the early region of polyoma DNA. These host-range mutants grow only in mouse cells transformed by defective polyoma virus. They also have the unselected property of being transformation negative (Staneloni *et al.*, 1977; Fluck *et al.*, 1977) and are thus termed host-range transformation mutants (hr-t). The hr-t site on the viral chromosome has now been mapped and found to be confined to the 5' end of the early RNA region, where small t is also encoded, and distinctly apart from the tsA class mutants (Feunteun *et al.*, 1976; Miller and Fried, 1976). Indeed, whereas cells infected with either wild type polyoma or the hr-t NG-18 mutant induce indistinguishable T antigens, cells infected with NG-18 mutant fail to induce a detectable t (Ito *et al.*, 1977).

Cells transformed by polyoma or SV40 viruses are stably altered in several metabolic and morphologic properties. Methods of selection of transformed cells are based on only some of the new properties acquired by the transformed cell. Transformants selected by different methods may vary in their properties. Polyoma and SV40 transformed cells do not

produce virus. Viral DNA synthesis or capsid proteins have never been detected in transformed cells (Todaro and Green, 1966a; 1966b; Henry *et al.*, 1966). However, the same viral coded or viral induced functions which are expressed 'early' in lytically infected cells are also expressed in transformed cells. They include surface antigens (Habel, 1961; Koch and Sabin, 1963; Coggin *et al.*, 1969), the membrane U antigen (Lewis *et al.*, 1969) and the nuclear T antigen (Black *et al.*, 1963; Habel, 1966).

T antigen is present in the nucleus of cells either transformed or productively infected by polyoma or SV40 viruses. As previously reported, T antigen is an early viral gene function and appears, from experiments with tsA mutants, (i) to control the replication of viral DNA in productively infected cells, (ii) to stimulate cellular DNA synthesis in transformed and productively infected cells, and, lastly, (iii) to be involved in the initiation and/or the maintenance of polyoma or SV40 induced cell transformation. The mechanisms by which T antigen exerts all these effects are at present only speculative.

With respect to its involvement in cell transformation, it has been reported that the A gene function is necessary for the establishment of transformation by both SV40 and polyoma viruses (Di Mayorca *et al.*, 1969; Eckhart, 1969; Kimura and Dulbecco, 1972; Tegtmeyer, 1972). Its continuous expression is necessary also for the maintenance of transformation in SV40 transformed cells selected in liquid medium (Martin and Chou, 1975; Tegtmeyer, 1975; Brugge and Butel, 1975). However, cells transformed by polyoma tsA mutants and selected by growth in soft agar do not require the continuous expression of the A gene for the maintenance of the transformed phenotype. It has recently been reported that polyoma transformed rat cells differed in their requirement for continuous A gene expression for maintenance of the transformed phenotype, when different methods of selection of transformants were used (Seif and Cuzin, 1977). Selection of

transformants in soft agar produces tsA transformed lines which maintain their transformed phenotype when shifted to the restrictive temperature, whereas transformants selected by their capacity to form dense foci on plastic in liquid medium, lose their transformed phenotype when shifted to the non-permissive temperature. It seems therefore, that two types of transformants are induced by polyoma virus. In one of them, selected in liquid medium, the transformed phenotype is primarily, if not exclusively, under the control of the A gene. In the other type of transformant, selected in soft agar, it appears as if a second gene controls transformation. That this is the case is suggested by recent experiments by Bouck *et al.* (1978). Viable SV40 mutants carrying deletions in the proximal part of the early DNA region (between positions 0.50 and 0.54), where little t antigen is specifically encoded, fail to form colonies in agar, but do form transformed colonies in liquid. These mutants complement A gene mutants for transformation.

A second gene other than A, involved in transformation had been defined also by the polyoma hr-t mutants described earlier (Benjamin, 1970). This view is reinforced by results from complementation experiments between tsA and hr-t mutants of polyoma virus. These two classes of mutants complement each other efficiently and symmetrically both for transformation and replication (Eckhart, 1977; Staneloni *et al.*, 1977; Fluck *et al.*, 1977). These results demonstrate that the tsA and hr-t mutant groups represent two distinct early gene functions in replication and transformation.

The persistence in polyoma or SV40 transformed cells of the T antigen, which is a virus coded protein, and of the other virus induced antigens is an indication that viral DNA sequences must be present in transformed cells. At least one complete copy of the viral genome must be present in some virus transformed cells since it has been possible to

rescue infectious SV40 or polyoma virus from them (Koprowski *et al.*, 1967; Watkins and Dulbecco, 1967; Fogel and Sachs, 1970; Summers and Vogt, 1971; Folk, 1973). Rescue of virus from transformed cells is achieved either by cocultivation, or by fusion, of the transformed cells with permissive cells. It is thus evident that viral DNA persists in SV40 and polyoma transformed cells from which virus is rescued. However, DNA sequences are also present in all the other virus transformed cell lines from which virus cannot be rescued. The detection of viral DNA sequences, which represent an extremely small fraction of the total amount of the DNA present in a cell, requires very sensitive techniques. DNA-RNA filter hybridisation (Gillespie and Spiegelman, 1965) and DNA-DNA reannealing kinetic techniques (Gelb *et al.*, 1971) have proved to be suited for the purpose. The former method measures the hybridisation between virus specific labelled c-RNA with total DNA extracted from transformed cells. Reconstruction experiments are then necessary to quantitate the amount of viral DNA in transformed cells. The latter method compares the rate of reannealing of high specific activity labelled viral DNA in the presence of total DNA from transformed cells and normal cells. Since the rate of reannealing of the labelled viral DNA is directly proportional to the total concentration of the viral DNA (Britten and Kohne, 1968; Wetmur and Davidson, 1968), a direct estimate of the amount of viral DNA per transformed cell is obtained. Though both methods are good for a qualitative detection of viral DNA, the DNA/RNA hybridisation method suffers from a systematic error in the reconstruction experiment (Haas *et al.*, 1972) which results in an overestimation of the amount of viral DNA per cell. When the error is corrected, the results obtained by the DNA/RNA hybridisation method are in good quantitative agreement with those obtained with the DNA/DNA reannealing kinetics. The persistence of viral DNA sequences in transformed cells can also be shown by the presence of viral m-RNA transcripts.

The first evidence for the persistence of viral DNA in papovavirus transformed cells was given by Benjamin (1966), who found that in cells of different species transformed by polyoma virus, a small fraction of the total RNA specifically hybridised to purified viral DNA. Further evidence for the presence of viral DNA in SV40 and polyoma transformed cells came later from the work of Westphal and Dulbecco (1968). The DNA-RNA hybridisation method was used and their results were confirmed in similar experiments by Tai and O'Brien (1969) and by Levine *et al.* (1970). Levine and co-workers, however, used an improved DNA-RNA hybridisation technique for the quantitation of the viral genome equivalents per transformed cell and reported values remarkably lower (between two and nine equivalents of SV40 DNA per transformed hamster cell) than those reported by Westphal and Dulbecco (about 60 SV40 equivalents per cell). A more exact quantitation of the amount of viral DNA per transformed cell was obtained by using DNA reannealing kinetics (Gelb *et al.*, 1971). With this method in four out of five SV40 transformed lines, an average of one SV40 DNA equivalent per transformed cell was present. Three SV40 DNA equivalents were found in the fifth cell line. Similar quantitative data were also obtained by Ozanne *et al.* (1973).

Sambrook *et al.*(1974) investigated which portion of the viral genome was present in SV40 transformed cells. Labelled SV40 DNA fragments, produced by sequential cleavage of SV40 DNA with restriction endonuclease EcoRI and HpaI, were used as viral probes for reannealing kinetics in the presence of DNA extracted from SV40 transformed cells. Most of the transformed cell lines examined contained the entire sequence of SV40 DNA, but different portions of the SV40 DNA were represented at different frequencies. Although many lines of SV40 transformed cells contain complete copies of the SV40 genome, the presence of the entire genome is not necessary for transformation. It is possible to transform cells with

fragments of SV40 DNA obtained by restriction endonuclease cleavage. The transforming fragments contain the entire early region of the viral DNA (Abrahams and van der Eb, 1975; Abrahams *et al.*, 1975). It has been shown also that though the whole genome of SV40 virus is present in transformed cells, only the 'early' region of the DNA is actively transcribed. The viral transcripts of the early lytic cycle are similar, if not identical, to the transcripts found in some transformed cell lines (Kamen *et al.*, 1974; Weinberg *et al.*, 1974; Khoury *et al.*, 1975; Kamen and Shure, 1976).

Concomitant with the report that viral DNA was present in transformed cells, reports appeared of the physical state of these viral DNA sequences within the cell. In the SV40 and polyoma cell lines previously investigated by Westphal and Dulbecco (1968), no molecules of free viral DNA in supercoiled circular form of any size were detected (Sambrook *et al.*, 1968). However, viral DNA corresponding to about 20 viral genome equivalents per cell, as judged by RNA/DNA hybridisation, was detected associated with cellular DNA from SV40 transformed cells, fractionated by sedimentation in alkaline sucrose gradients. It was concluded that the viral DNA was integrated into the cellular DNA by alkali-stable bonds (though it was not possible to rule out the possibility that oligomeric forms of SV40 DNA co-sedimented with high molecular cell DNA in the alkaline sucrose gradients).

Botchan and McKenna (1973) investigated the integration of SV40 DNA into high molecular weight DNA from SV40 transformed mouse cells using the restriction endonuclease EcoRI. The total DNA from SV40 transformed cells was first digested with the enzyme, and then fractionated by agarose gel electrophoresis. The SV40 DNA sequences were localised on the gel by hybridisation with labelled c-RNA. SV40 DNA is cleaved by EcoRI at only one site. Two fragments of SV40 DNA were found on the gel and the sum of their molecular weights was higher than the molecular weight of SV40.

This result suggested that the viral DNA fragments were covalently linked to cellular DNA.

Investigations on the presence and the state of viral DNA sequences in polyoma transformed cells have revealed a more complex situation. Manor *et al.* (1973) detected about thirty polyoma DNA equivalents per cell in two clones derived from an inducible line of polyoma transformed rat cells. Of these, only six to nine DNA equivalents per cell were integrated, as estimated from hybridisation of c-RNA with DNA purified by sedimentation in alkaline glycerol gradients. The presence of the free polyoma DNA molecules, as well as that of the integrated ones, was further analysed in different cell lines of polyoma transformed rat cells (Prasad *et al.*, 1976; Zouzias *et al.*, 1977). These transformed rat cells yielded virus upon fusion with permissive cells. They contained from twenty to fifty copies of non-integrated viral DNA equivalents per cell, and the presence of free viral DNA molecules was a stable property of the transformed cells. Most of the free polyoma DNA molecules were localised in the nuclei of the transformed cells, had a circular supercoiled conformation, and were infectious. The maintenance of the free polyoma DNA molecules was tsA dependent. From six to eight equivalents of polyoma DNA were found integrated in cellular DNA from transformed cells, as judged by reannealing kinetics of labelled polyoma DNA in the presence of transformed cellular DNA purified by the network technique (Varmus, 1973). The amount of integrated viral DNA was constant and tsA independent. *In situ* hybridisation experiments showed that only a fraction of the transformed cells contained free polyoma DNA. It was concluded that spontaneous induction of viral DNA replication was occurring at a low level in the transformed cell population and that free viral DNA molecules were likely to have arisen from the integrated ones. Very similar results were also reported for polyoma transformed hamster cells (Folk and Bancuk,

1976). There has been only one report of free, circular, supercoiled infectious SV40 DNA recovered from SV40 transformed cells (Daya-Grosjean *et al.*, 1975).

Information about the site, the specificity and the pattern of integration of viral DNA in transformed cell DNA is at present scarce and limited to the SV40 system. The experiments by Gelb and Martin (1973) indicated that viral DNA was associated preferentially with the non-repeated fraction of the cell DNA in SV40 transformed cells. The distribution of the SV40 DNA sequences between unique and repetitive host DNA fractions varied from one cell line to another and suggested that there was more than one site of SV40 DNA integration.

Later reports by Ketner and Kelly (1976) and Botchan *et al.* (1976) supported this conclusion. Both groups independently investigated the arrangement of SV40 DNA sequences in the DNA of several lines of SV40 transformed cells using the same experimental approach. The transformed cell DNA from each line was digested with various restriction enzymes, electrophoresed on agarose gels, transferred from the gel to nitrocellulose paper as described by Southern (1975) and hybridised *in situ* with labelled SV40 c-RNA or DNA. This method makes it possible to locate the restriction fragments containing the viral DNA and estimate their molecular weight. From the analysis of five lines of mouse cells independently transformed by SV40, Ketner and Kelly (1976) concluded that the structure of the integrated SV40 DNA and/or its location in the host genome was different in each transformed line. Botchan *et al.*(1976) analysed very extensively the viral DNA sequences in eleven lines of SV40 transformed rat cells. Seven of those lines, containing multiple copies of SV40 DNA, gave complex patterns after digestion with three restriction enzymes. It was concluded that in these seven lines there were multiple insertions of SV40 DNA. The other four cell lines contained

one single insertion of viral DNA at different locations on the cell chromosome. Also, the junction between cell and viral DNA involved different sequences of the SV40 DNA in each line. Thus integration of SV40 DNA in transformed cells appears to occur at several sites in both cellular and viral DNA.

Integration of the viral genome cannot be completely nonspecific if the 'early' region of the viral is preserved during the integration event. Also the fact that virus can be rescued from some transformed cell lines indicates that the recombinational event leading to integration must preserve all of the viral genetic information. From experiments on hybrid cells obtained by fusion of SV40 transformed human cells and monkey cells, permissive for SV40, Croce and co-workers suggested that SV40 DNA integrates specifically in one morphologically identifiable human chromosome (Croce *et al.*, 1973; 1974). Taken together with the above results (Botchan *et al.*, 1976) this would mean that all the different sites of integration of SV40 DNA in the cell DNA are clustered in one specific chromosome.

It is not known what role is played by the integration of viral DNA in the events leading to cell transformation. Integration of viral genes into cellular genes may provide a stable environment for the continuous systhesis of virus specific products either leading to cell transformation or necessary to maintain the cell transformed phenotype. It can be speculated that the integration of viral DNA into regulatory cellular genes could also induce irreversible mutations in the host genes leading to the acquisition by the cell of some new 'transformed' properties. The continuous expression of the integrated genes might also be necessary for full transformation.

There is evidence that SV40 and polyoma viruses integrate their DNA into the DNA of host cells lytically or abortively infected as well as into the DNA of transformed cells. Several groups have investigated the fate of the infecting viral DNA in abortively infected cells. Hirai *et al.* (1971) found that in SV40 infected chinese hamster ovary cells viral DNA sequences became associated in an alkali-stable form with cell DNA early (10-20 h) after infection, and that the association was not affected by cytosine arabinoside. Integrated viral DNA was also detected later in infection (30 h post infection). The fate of infecting SV40 DNA in mouse cells abortively infected by SV40 was investigated by Collins and Sauer (1972). Infection was achieved with naked SV40 form I DNA instead of with virions. The supercoiled infecting viral DNA was converted first to slower sedimenting forms, probably open circular or linear molecules. Within 2 days p.i. viral DNA appeared cosedimenting with cellular DNA in alkaline sucrose or CsCl gradients and thus probably in an integrated form. Up to 1,200 equivalents of SV40 DNA per cell were integrated at 48 h post infection. Similarly, integration of polyoma DNA in abortively infected hamster and human cells has been shown (Babiuk and Hudson, 1972).

Integration of viral DNA into cellular DNA apparently occurs in cells productively infected by polyoma or SV40. In a permissive cell system the detection of integrated viral genomes becomes complicated by the high amount of the viral DNA replicated and accumulated late in infection. Nonspecific trapping of free monomeric virion DNA in the bulk of fast sedimenting cell DNA can occur, but it can be accounted for by adequate controls. A more problematic contamination of fast sedimenting cell DNA results from multimeric, circular or linear viral DNA forms of very high molecular weight arising during replication of the viral DNA

(Jaenisch and Levine, 1971; Martin *et al.*, 1976). One way of circumventing these problems is to infect the cells in the presence of inhibitors of DNA replication, which do not seem to inhibit integration and allow the early events of infection to take place. It could be objected that these conditions reproduce artificially those of non-permissive cells and might influence the occurrence of integration. However, this approach has been adapted by Hirai and Defendi (1972) and Ralph and Colter (1972) in the investigation of the integration of SV40 and polyoma DNA during productive infection. Ralph and Colter (1972) infected BUdR labelled mouse cells with polyoma virus in the presence of the DNA synthesis inhibitor 5-fluorodeoxyuridine. Following fractionation of 'heavy' cell DNA and 'light' viral DNA in CsCl equilibrium gradients, 8-15 polyoma genome equivalents per cell were detected in the BUdR containing DNA at 14 h post infection. Hirai and Defendi (1972) infected monkey cells with a low multiplicity of SV40 virus in the presence of the inhibitor of DNA synthesis cytosine arabinoside. They reported the appearance of viral DNA sequences in both the Hirt pellet (Hirt, 1967) and in DNA that sedimented rapidly in alkaline sucrose from about 20 h post infection.

An estimate of the viral DNA integrated in the DNA of cells productively infected by SV40 was attempted by Hölzel and Sokol (1974) without the use of an inhibitor of viral DNA synthesis. The cell DNA from the infected cells was purified by a multistep procedure consisting of (i) precipitation by the Hirt (1967) method, (ii) centrifugation in alkaline sucrose gradients, (iii) equilibrium centrifugation in ethidium bromide - CsCl gradients, and finally (iv) another centrifugation in alkaline sucrose gradients. The efficiency of purification of each step was determined from the amount of contamination by the free viral DNA in a parallel control experiment. Up to 20,000 copies of SV40 DNA per cell

were associated with cell DNA purified in this way. Association of viral DNA with cell DNA was detected starting from 24 h post infection.

This value for the integrated viral DNA appears excessive and raises the question of whether the cell DNA, though thoroughly purified by conventional methods, contained unusual viral DNA contaminants. Indeed, oligomeric SV40 DNA forms are detected at late times in infection (at which the above experiments were performed) (Jaenisch and Levine, 1971; Martin *et al.*, 1976), and can account for more than 10 percent of the viral DNA in monkey cells productively infected by SV40 at 70 h post infection. These viral DNA oligomers, dimers up to hexameres, sediment with the DNA pellet during Hirt (1967) separation of cell from viral DNA, and sediment with cellular DNA during alkaline sucrose gradient sedimentation. It is therefore very likely that most of the viral DNA found associated with purified cellular DNA in the experiments of Holzel and Sokol (1974) was due to contamination by free viral DNA of the type described, though it is possible that some viral DNA is truly integrated, since SV40 DNA has been shown to integrate also in absence of its replication (Hirai and Defendi, 1972). The biological significance of integration during lytic infection is unknown. There are at present no data suggesting that integration plays any obligatory role in the infectious life cycle of the virus. Nothing is known about the relationship between the integration event and the other events induced by the virus during the early stage of infection. Apparently cellular DNA synthesis, which is induced in permissive and non-permissive cells by SV40 or polyoma infection, is not a prerequisite for integration of viral DNA (Hirai/Robb and Defendi, 1974). Integration of viral DNA during lytic infection could be the result of a cellular recombination system, enhanced by the presence of large amounts of viral DNA. Integration of

viral DNA into cellular DNA during productive growth may cause alterations in the regulation of transcription and replication of the host cell genome and contribute to cytopathic effects and cell death.

Integration during lytic infection provides a plausible explanation for the occurrence of SV40 variants, whose genomes contain host DNA substitutions (Lavi and Winocour, 1972; Martin *et al.*, 1973). The genomes of these variants could have arisen by defective excision of viral DNA integrated into cellular DNA. Integration of viral DNA into cell DNA may also explain the occurrence of RNA transcripts containing covalently linked host specific and virus specific segments, detected in cells productively infected or transformed by SV40 or polyoma (Wall and Darnell, 1971; Weinberg *et al.*, 1972).

Adenoviruses

The adenoviruses are non-enveloped icosahedral DNA viruses which multiply in the nuclei of infected cells. More than eighty distinct serotypes have been isolated from animals of different species (Green, 1970; Tooze, 1973) and over thirty from humans. Adenoviruses cause mild respiratory illness in man.

As compared to papovaviruses, adenoviruses are larger and more complex. Adenoviruses share with the papovaviruses many properties in their mode of infection. They replicate productively and with high yields in permissive cells. Human and non-human adenoviruses are also able to transform non-permissive cells in culture and cause tumours *in vivo* in suitable animals. Like papovaviruses, adenoviruses provide a good experimental system for the investigation of (i) the replication cycle of DNA tumour viruses, (ii) the mechanism of cell transformation and possibly (iii) some aspects of the molecular biology of the eukaryotic host cell.

Recent years have seen very fast advances in understanding of the replicative machinery of adenoviruses and in identification of the virus-coded transforming function(s).

Most of the information about adenovirus DNA structure and replication and adenovirus induced transformation have been obtained from work with the human serotypes 5 (Ad5) and 2 (Ad2). One avian serotype, chick embryo lethal orphan (CELO) virus has been investigated with respect to the structure and the replication of its DNA, and results comparable to those from human serotypes have been obtained. The adenovirus genome is a linear duplex DNA molecule of molecular weight ranging from 20 to 28 x 10^6. The DNA of the human serotypes has a molecular weight between 20 and 25 x 10^6 (Green *et al.*, 1967; Doerfler and Kleinschmidt, 1970), and that of CELO virus 28 x 10^6 (Robinson *et al.*, 1973).

The adenovirus chromosome can be isolated from purified virions as a DNA-protein complex (Robinson *et al.*, 1973; Robinson and Bellett, 1974; Sharp *et al.*, 1976; Rekosh *et al.*, 1977). Procedures of extraction of viral DNA from purified virions which exclude the use of proteolytic enzymes, yield circular and oligomeric adenovirus DNA molecules. The circular molecules are generated by a protein-protein link between the termini and the ends of the DNA molecules are not covalently joined. A protein, of approximate molecular weight 55,000, is covalently joined to the 5' end of each strand of the duplex adenovirus DNA molecule (Robinson and Bellett 1974; Sharp *et al.*, 1976; Rekosh *et al.*, 1977). It appears that this protein is also bound to the ends of intracellular replicative forms of adenovirus DNA and that it might play some role in the replication of the viral DNA (Girard *et al.*, 1977; Stillman and Bellett, 1978; Robinson *et al.*, 1978).

Adenovirus DNA is a non-permuted linear molecule containing neither cohesive ends nor direct terminal repetitions (Kimes and Green, 1970; Doerfler and Kleinschmidt, 1970; Younghusband and Bellett, 1971; Robinson *et al.*, 1973). The absence of these features suggests that adenovirus DNA cannot form double stranded circular or concatemeric molecules by recombination or by base pairing of complementary DNA single stranded ends and must therefore replicate without such intermediates. Replicative intermediates of adenovirus DNA are linear molecules containing extensive stretches of single stranded DNA. For this reason the replicative DNA has a greater sedimentation rate in neutral sucrose gradients and a higher buoyant density in CsCl gradients than mature adenovirus DNA (Pearson and Hanawalt, 1971; Sussenbach and van der Vliet, 1972; Bellett and Younghusband, 1972; Pettersson, 1973, Pearson, 1975). Replication of adenovirus DNA is semiconservative and proceeds via a displacement mechanism (Pearson and Hanawalt, 1971; Bellett and Younghusband, 1972; van der Vliet and Sussenbach, 1972; Sussenbach *et al.*, 1972; van der Eb, 1973; Ellens *et al.*, 1974). Origins and termini of replication occur at both ends of the DNA molecule (Tolun and Pettersson, 1975; Shilling *et al.*, 1975; Horwitz, 1976; Lechner and Kelly, 1977).

The major problem with a mechanism of replication involving linear intermediates is the conservation of the genetic information at the ends of the molecule. All known DNA polymerases require a 3'-OH group as a primer, which might be provided by a short RNA sequence. Removal of the RNA primer from the 5' end of a linear DNA molecule leaves a single stranded parental 3' end with no complementary progeny sequence. How synthesis of the 5' end is completed to yield mature fully double stranded linear DNA molecules has yet to be discovered. The ends of the adenovirus DNA molecule have two unique structural features as well as the 55k terminal protein: an inverted repeated sequence, 100-140 nucleotides long (Wolfson and Dressler, 1972; Garon *et al.*, 1972; Roberts *et al.*, 1974;

Robinson and Bellett, 1975), and a possible palindromic sequence located some distance from the termini (Padmanaban *et al.*, 1976). The nucleotide sequence at the termini of Ad5 DNA, including the inverted terminal repeat, has been determined (Steenbergh *et al.*, 1977; Arrand and Roberts, personal communication). No palindromic sequence is present in the part that has been sequenced to date (about 200 base pairs in). The functions of the inverted terminal repeat and of the protein on the 5' ends have not been definitely established. There is some evidence that the terminal protein is involved in priming replication at the 5' ends of adenovirus DNA (Rekosh *et al.*, 1977; Stillman and Bellett, 1978; Robinson *et al.*, 1978). It has also been proposed that the inverted terminal repetition allows the formation of a single-stranded circle with a duplex 'panhandle' from the parental strand which is displaced, and that the duplex section of this molecule is required to initiate replication of the second progeny strand (Lechner and Kelly, 1977).

Physical maps of the DNA of human adenovirus type 5 (Ad5) and type 2 (Ad2) have been obtained by the use of restriction endonucleases to cleave the viral DNA into a discrete number of fragments which can be linearly ordered on the viral DNA molecule. The enzymes most commonly used have been EcoRI, HpaI, HindIII and BamHI (Pettersson *et al.*, 1973; Mulder *et al.*, 1974; Sharp *et al.*, 1974). As described for papovaviruses DNA, the adenovirus DNA map is divided into 100 units. Position 0 is by convention at the left end of the DNA molecule in the region with higher G + C content, which was identified by partial denaturation mapping (Doerfler and Kleinschmidt, 1970). Also 'early' and 'late' regions can be defined with respect to their transcription prior to or after the onset of viral DNA replication respectively.

The two complementary strands of the adenovirus DNA molecule are separable. One strand (the H or heavy strand) has greater affinity for poly (U, G) than the other strand (the L or light strand). They can thus be separated by centrifugation in CsCl buoyant density gradients (Landgraf-Leurs and Green, 1971; Tibbetts *et al.*, 1974). During adenovirus infection the H and the L strands are transcribed leftward (l) and rightward (r) respectively. One strand of the adenovirus DNA molecule can thus be defined as the H or l strand, while the other strand is the L or r strand. Early m-RNA sequences are copied from four widely spaced regions of the adenovirus DNA molecule, two on each strand (Sharp *et al.*, 1974; Tibbetts and Pettersson, 1974; Flint *et al.*, 1976; Ortin *et al.*, 1976). m-RNA isolated at late times in infection, contains all the early sequences in addition to exclusively late sequences which account for all the remaining information of the genome. The majority of the late m-RNA is transcribed from the r strand (Figure 3).

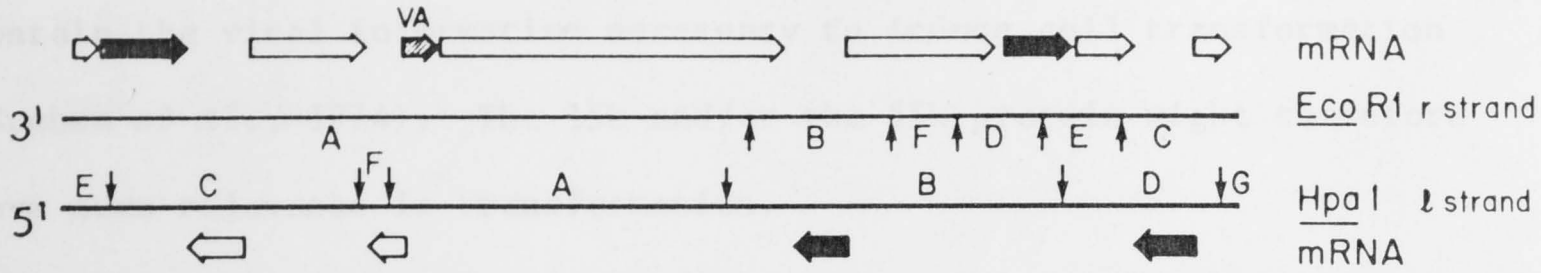
It is possible to map directly on the DNA molecule viral polypeptides by *in vitro* translation of m-RNA species selected by hybridisation to restriction fragments of adenovirus DNA (Lewis *et al.*, 1975; 1976). The same polypeptides can also be detected *in vivo* by radioactive labelling (Lewis, 1974; Harter *et al.*, 1976; Harter and Lewis, 1978). *In vitro* translation of early m-RNAs produces six non-structural polypeptides (Lewis, 1976) while *in vitro* translation of late m-RNA species yields virion structural polypeptides (Lewis, 1975; Figure 3). The functions of some of the early polypeptides appear to be crucial both for the replication of the viral DNA and virus induced cell transformation. A 40k to 55k and a 15.5k protein are translated *in vitro* from early m-RNA selected by hybridisation of the left-end sequences of Ad2 DNA (Lewis *et al.*, 1976). It has been suggested that the 55k protein may be the protein found covalently attached to the 5' ends of adenovirus DNA (Robinson and Bellett,

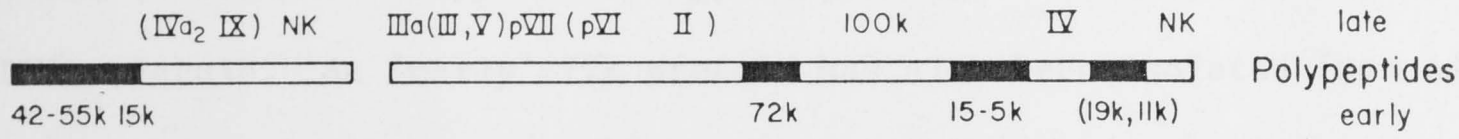
FIGURE 3: Topography of the adenovirus genome (adapted from Flint, 1976)

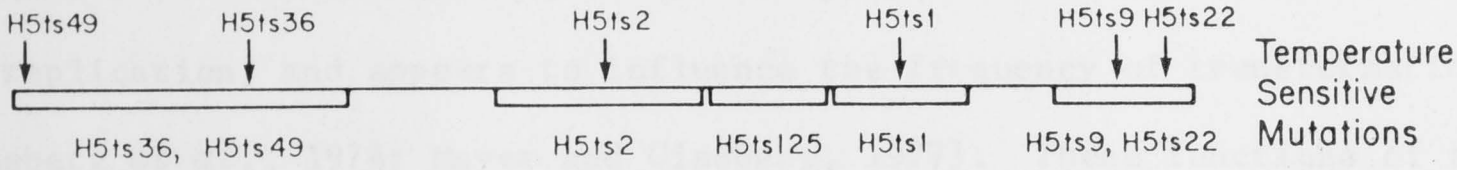
The adenovirus genome is shown by horizontal lines, 0-100 units, in each part of the figure. In the map of mRNA sequences, the two lines represent the r and l strands of Ad2 DNA; the vertical lines indicate sites of cleavage by EcoRI and HpaI. The arrows above and below the genome depict the regions to which mRNA is complementary; genes for the non-messenger RNA, VA-RNA, are indicated by the stippled area. In this, and the map of polypeptides shown below, solid lines indicate early viral gene products, whereas open areas show late products. In the polypeptide map, parentheses indicate uncertainty in order.

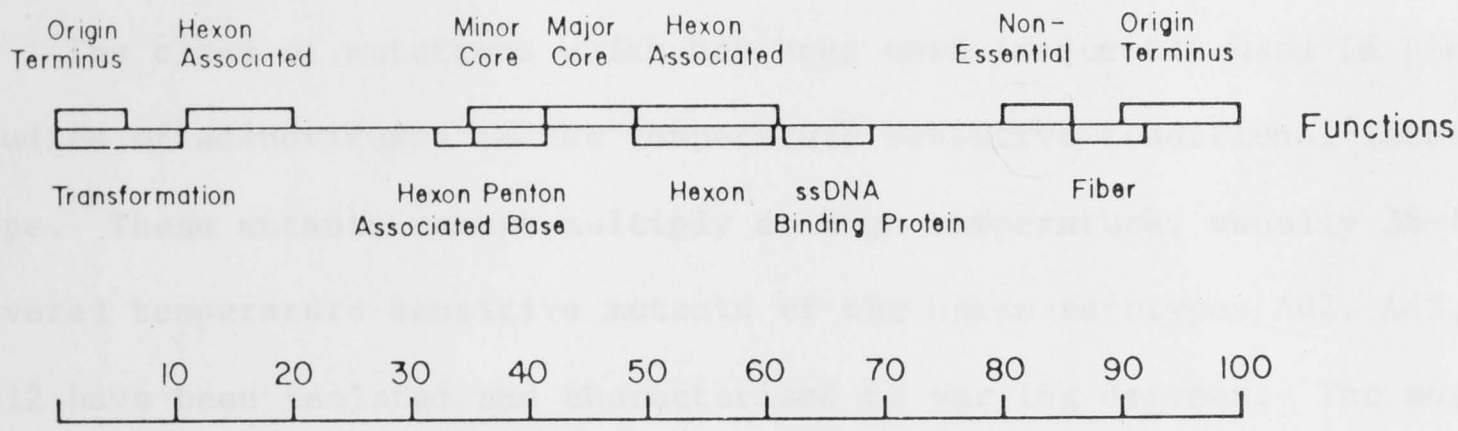
In the map of temperature-sensitive mutations of Ad5, the relative locations based on recombination frequencies are indicated above the line. Below are shown the limits within which physical mapping places the sites of mutation. Mutants H5 ts9 and H5 ts22 fail to synthesise fiber, whereas H5 ts2 makes reduced quantities of hexon at the restrictive temperature. H5 ts1 is deficient in transport of hexon into the nucleus. The phenotype of H5 ts49 is not well defined.

The final part of this figure summarises the preceding information in the form of a functional map of the adenovirus genome, in which the regions to which the various functions have been located are indicated. No information about strand assignment is intended. It also shows the regions in which replication of DNA is initiated and terminated, and a region whose expression is not essential for growth of the virus in tissue culture.


VA
mRNA
3'
Eco R1 r strand
A
B
F
D
E
C
E
C
F
A
B
D
G
5'
Hpa 1 l strand
mRNA


(IVa2 IX) NK
IIIa(III,V)pVII (pVI II)
100k
IV
NK
late
42-55k 15k
72k
15-5k
(19k, 11k)
Polypeptides
early


H5ts49
H5ts36
H5ts2
H5ts1
H5ts9 H5ts22
H5ts36, H5ts49
H5ts2
H5ts125
H5ts1
H5ts9, H5ts22
Temperature Sensitive Mutations


Origin Terminus
Hexon Associated
Minor Core
Major Core
Hexon Associated
Non-Essential
Origin Terminus
Functions
Transformation
Hexon Penton Associated Base
Hexon
ssDNA Binding Protein
Fiber
10
20
30
40
50
60
70
80
90
100

1974; Rekosh *et al.*, 1977). The terminal left-end fragment of the Ad2 DNA corresponding to 7 percent of the viral genome has been shown to contain the viral information necessary to induce cell transformation (Graham *et al.*, 1974). The 15k and/or the 55k protein might therefore have some relevance in transformation.

A 72k protein is translated from early m-RNA mapping between positions 66.5 and 61.6 approximately, to the right of the centre in the Ad2 DNA molecule. An 'early' 72k protein has also been isolated from Ad5 infected cells. This protein in its native form binds to single stranded DNA (van der Vliet and Levine, 1973). It plays an essential role in viral DNA replication, and appears to influence the frequency of transformation (Ginsberg *et al.*, 1974; Mayer and Ginsberg, 1977). These functions of the 72k protein will be discussed further in following sections.

The class of mutations which has been most frequently used in genetic studies of adenoviruses is the temperature sensitive conditional lethal type. These mutants cannot multiply at high temperature, usually 38-40°C. Several temperature sensitive mutants of the human serotypes Ad2, Ad5, and Ad12 have been isolated and characterised to varying degrees. The most detailed genetic analysis has been done on Ad5. A genetic map of Ad5 DNA based on recombination frequencies between members of different complementation groups has been constructed (Williams, 1974). However, the genetic map simply orders the various viral functions in relation to one another. A physical map of the Ad5 ts mutants has also been constructed and oriented with the genetic map by the use of an elegant technique devised by Grodzicker *et al.*(1974). They isolated wild type recombinants from interserotypic crosses between temperature sensitive mutants of Ad5 and mutants of the non-defective Ad2-SV40 hybrid Ad2^{+}ND1. The positions of Ad2^{+}ND1 and of Ad5 DNA sequences in recombinant genomes can be determined by analysis of their DNA with restriction endonucleases.

The crossover points in the molecules yielding wild type recombinants could be established. The physical map correlates well with the genetic map (Figure 3).

Most of the Ad5 ts mutants characterised so far are defective in a late function, i.e., in proteins, mostly structural, systhesised at late stages of infection following the initiation of viral DNA replication. However, a limited number of early ts mutants defective in DNA replication have also been isolated. These mutants do not synthesise viral DNA at the non-permissive temperature and are thus called DNA negative mutants. Since the expression of early viral functions is essential for viral DNA replication and for virus induced transformation, the early adenovirus ts mutants provide very useful tools for the identification of the viral products required for DNA replication and transformation. Again, the results obtained with Ad5 will be described as they are the most comprehensive. The DNA negative, ts mutants of Ad5 fall into two complementation classes, one represented by Ad5 ts125 (Ensinger and Ginsberg, 1972) and the other by Ad5 ts36 and Ad5 ts37 (Williams *et al.*, 1974) and Ad5 ts149 (Ginsberg *et al.*, 1974).

At the non-permissive temperature both types of mutants fail to synthesise viral DNA (Ginsberg *et al.*, 1974; Levine *et al.*, 1974; van der Vliet and Sussenbach, 1975) and do not produce capsid or other viral proteins (Ensinger and Ginsberg, 1972; Russell *et al.*, 1974). Both Ad5 ts125 and ts36 replicate normally at the permissive temperature. When cells infected with the mutants are growing at the permissive temperature and then shifted up to the restrictive temperature, the rate of DNA replication of Ad5 ts125 declines rapidly (within 30 min.), whereas that of Ad5 ts36 declines over 4-6 h after the temperature shift (Williams *et al.*, 1974; Levine *et al.*, 1974; van der Vliet and Sussenbach, 1975). Ad5 ts149, though belonging to the same complementation group as

Ad5 ts36, reduces its DNA replication with 1-2 h from the time of the shift. It has been suggested that both Ad5 ts36 and ts125 gene products are necessary for the initiation of viral DNA replication (van der Vliet and Sussenbach, 1975), and the Ad5 ts125 gene product may function in elongation of viral DNA chains as well as in initiation of rounds of viral DNA synthesis (van der Vliet *et al.*, 1977). The gene product of the Ad5 ts125 mutant has been shown to be the early 72k protein encoded to the right of centre on the Ad5 DNA molecule. This protein is equivalent to the 72k early protein described for Ad2. It binds specifically to single stranded DNA (van der Vliet and Levine, 1973). The Ad5 ts125 mutant has been mapped on the portion of the viral DNA which codes for the 72k protein (Lewis *et al.*, 1976). Moreover Ad5 ts125 produces a protein thermolabile for continuous binding to single stranded DNA at the non-permissive temperature (Levine *et al.*, 1974; van der Vliet and Sussenbach, 1975).

The defective Ad5 ts36 protein has not yet been identified. The corresponding gene has been located on the left hand side of the Ad5 DNA molecule. Most interestingly, both Ad5 ts36 and Ad5 ts125 mutants have altered ability to transform cells, as compared with wild type Ad5. These properties and their implications will be discussed in a following section concerned with transformation by adenoviruses and in one experimental chapter.

Adenoviruses with oncogenic potential have been isolated from animals of different species; simian, bovine, avian and human (Hull *et al.*, 1965; Sarma *et al.*, 1965; Darbyshire, 1966). Thirty-one adenovirus serotypes have been isolated from humans, and they have been subdivided into three subgroups, A, B and C on the basis of their oncogenicity.

The degree of oncogenicity is determined by the inoculum of virus required, the time, and the frequency of appearance of tumours in newborn hamsters. On this basis group A (Ad12, 18 and 31) and group B (Ad3, 7, 11, 14, 16, 21) adenoviruses are defined as 'highly' and 'weakly' oncogenic respectively. Group C (Ad1, 2, 5, 6) adenoviruses are defined as non-oncogenic since they cannot induce tumours in hamsters unless the animals are immunosuppressed. They do, however, transform rodent cells in culture (Freeman *et al.*, 1967; McAllister *et al.*, 1969).

Adenoviruses within each group are closely related and share 60-100 percent of their base sequences, while those of different groups share 5-15 percent of their sequences (Piña and Green, 1968; Fujinaga *et al.*, 1969).

Transformation of non-permissive cells by adenoviruses can be achieved by infection with either intact virions or naked viral DNA (Graham and van der Eb, 1973). Non-infectious fragmented adenovirus DNA also retains its transforming capacity (Mayne *et al.*, 1971; Graham *et al.*, 1974). Adenovirus transformed cells present stable morphological and metabolic changes, as do the papovavirus transformed cells. However, cells transformed by adenoviruses have a characteristic morphology, and cells which have been transformed by adenoviruses or papovaviruses are distinguishable (Strohl *et al.*, 1967). Also adenovirus transformed cells are selected from a population of untransformed cells by conditions that favour the growth of cells with altered properties, in this case growth in medium containing 0.1 mM Ca^{++} (Freeman *et al.*, 1967). The definition of cell transformation is, once again, relative to the method of selection used.

Adenovirus transformed cells do not produce virions or viral structural proteins. Virus particle production has never been induced in adenovirus transformed cells by any of the methods of virus induction

successfully used with papovavirus transformed cells. Several lines of evidence indicate, however, that adenovirus DNA sequences are present and are expressed in transformed cells.

That adenovirus DNA is present and expressed in transformed cells is indicated by the appearance of virus specific antigens. Among these are the TSTA (transplantation antigen) surface antigen (Sjogren *et al.*, 1967) and the T (tumour) antigen. T antigen is a protein(s) usually detected by antisera obtained from animals bearing adenovirus induced tumours (Huebner *et al.*, 1963). T antigen is present in cells transformed by human adenoviruses from each group. The T antigens induced by adenoviruses from different groups are serologically distinct. T antigen is also induced 'early' during lytic infection with adenoviruses (Hoggan *et al.*, 1965) and is detected in both nucleus and cytoplasm.

Transformation experiments with specific restriction endonuclease fragments of Ad2, Ad5, and Ad12 DNA located the transforming DNA segment within the extreme left end 7 percent of the adenovirus DNA molecule (Graham *et al.*, 1974; Shiroki *et al.*, 1977). T antigen is present in cells transformed with the left end fragment of adenovirus DNA (Shiroki *et al.*, 1977) and must therefore be encoded in, or induced by, these sequences of the viral DNA. The T antigen characteristic of group C adenoviruses has been identified as a 58k protein (Levinson and Levine, 1977). Two polypeptides, one of 55k, are translated *in vitro* from m-RNA isolated from group C adenovirus transformed rat cells which are T antigen positive and express only the early transcripts mapping near the left end of the genome (Lewis *et al.*, 1976). Also Gilead *et al.* (1976) detected a 15k and a 53k protein in Ad2 infected cells by immunoprecipitation with three antisera expected to contain antibodies against the Ad2 transforming proteins(s). Therefore, one (or both) of these proteins must be a candidate for T antigen. The 55k early protein translated

in vitro from Ad2 early m-RNA (Lewis *et al.*, 1976) could be the 55k protein covalently attached to the ends of adenovirus DNA (Rekosh *et al.*, 1977).

The class of 'early' temperature sensitive mutants of Ad5, epitomised by Ad5 ts36, has been mapped at the extreme left end of the Ad5 DNA genetic map (Williams *et al.*, 1974). Physical mapping techniques locate the Ad5 ts36 gene within the left hand 30 per cent (Sambrook *et al.*, 1975). Experiments to locate the mutation more accurately are in progress. The Ad5 ts36 mutant fails to transform non-permissive rat cells at the restrictive temperature, whereas it transforms the same cells with the same frequency as wild type Ad5 at the permissive temperature (Williams *et al.*, 1974). Cells transformed by Ad5 ts36 at the permissive temperature, maintain the transformed phenotype when shifted to high temperature (Williams *et al.*, 1974). The Ad5 ts36 gene product thus appears to be necessary for the establishment and not for the maintenance of the transformed state. The Ad5 ts36 gene product(s) is necessary also for initiation of viral DNA replication, as previously mentioned.

Additional evidence that the adenovirus transforming gene(s) lies to the left part of the DNA map is given by the Ad5 host range (hr) mutants (Harrison *et al.*, 1977; Graham *et al.*, 1978). These mutants do not replicate efficiently in human cell lines, such as KB or HeLa cells, but grow efficiently in embryonic human kidney (HEK) cells transformed by Ad5. Transformation of HEK cells, normally permissive to Ad5, is achieved by exposing the cells to sheared Ad5 DNA (Graham *et al.*, 1977). The hr mutants isolated to date fall into two complementation groups (I and II). Both groups of mutants are defective in transformation (Graham *et al.*, 1978). Group I hr mutants are able to initiate abnormal transformation of rat embryo kidney cells, while group II mutants cannot initiate transformation at all. Complementation and recombination experiments

between the Ad5 hr mutants and the 'early' Ad5 ts mutants indicate that their respective mutations are in distinct cistrons all residing near the left end of the Ad5 DNA map. The group I hr mutation appears to map within or close to the extreme left hand 4.4 percent, while group II hr mutations lie within the 25 percent left end of Ad5 DNA at a position not yet located more exactly (Graham *et al.*, 1978).

Virus specific nuclear and polysomal RNA have been isolated from cells transformed by adenoviruses (Green *et al.*, 1970). The genes transcribed in transformed cells correspond to the genes transcribed early in productive infection (Green *et al.*, 1970; Bachenheimer and Darnell, 1976; Chinnandurai *et al.*, 1976; Ortin *et al.*, 1976). Late viral genes are not transcribed in transformed cells. Large RNA molecules containing both cellular and viral sequences have been isolated from nuclei of adenovirus transformed cells (Tseui *et al.*, 1972; Wall *et al.*, 1973).

Adenovirus DNA sequences persisting in transformed cells have been detected directly either by DNA-DNA reannealing kinetics (Gelb *et al.*, 1971) or by DNA-RNA hybridisation (Westphal and Dulbecco, 1968) as previously described. Green *et al.* (1970) found that rat cells transformed by Ad2 and hamster cells transformed by Ad12 or Ad7, contained multiple copies of viral DNA. These estimates of the amounts of viral DNA were obtained by RNA-DNA hybridisation experiments. Pettersson and Sambrook (1973) found that, when the DNA-DNA reannealing kinetic technique was used, only one copy of Ad2 DNA per diploid amount of cell DNA was detected in Ad2 transformed rat cells.

By the use of restriction endonuclease cleavage products of viral DNA as probes in DNA-DNA reannealing kinetics it has been shown that several lines of rat cells transformed by Ad2 and Ad5 contain several copies of fragments of the viral DNA, ranging from about 10 to 90 percent

of the genome (Gallimore *et al.*, 1974; Sharp *et al.*, 1974). All cell lines contained DNA sequences from the left end 14 percent of the adenovirus DNA molecules and in several cases they were the only viral DNA sequences present (Gallimore *et al.*, 1974). In agreement with these findings, the viral m-RNA sequences from these Ad2 and Ad5 transformed rat cells were shown to be transcribed only from the viral DNA sequences which are expressed in the early phase of Ad5 infection (Sharp *et al.*, 1974; Flint *et al.*, 1976). Once again it is confirmed that the left end of the Ad2 and Ad5 genomes is necessary and can be sufficient to establish transformation.

On the other hand, rat cells transformed by the mutant Ad5 ts125 at the non-permissive temperature appear to contain multiple copies of the whole Ad5 genome (Mayer and Ginsberg, 1977). Ad5 ts125 transforms rat cells at the non-permissive temperature with frequency far higher than Ad5 wild type virus. The frequency of transformation by Ad5 ts125 at that temperature is comparable to that by the 'highly' oncogenic Ad12 (Williams *et al.*, 1974). It is interesting in this respect that most hamster cells transformed by Ad12 contain viral DNA sequences accounting for 90 to 100 percent of the viral genome (Fanning and Doerfler, 1976; Green *et al.*, 1976).

Viral DNA sequences have also been detected in cells transformed by non-human adenoviruses. In hamster cells transformed by the avian adenovirus CELO, viral DNA sequences homologous to part of the viral DNA were found (Bellett, 1975; May *et al.*, 1975).

Two cell lines derived from CELO virus-induced hepatomas and one from transformed hamster skin contained viral DNA sequences accounting for only a portion of the CELO virus DNA as detected by reannealing kinetics with restriction fragments of CELO virus DNA (May *et al.*, 1975; May *et al.*, 1978). Two transformed cell lines contained copies of most

of the viral genome, while the other lacked more than 50 percent of the viral sequences. Though a complete map of CELO virus DNA is not yet available, the DNA end fragments have been identified, after EcoRI cleavage of the CELO virus DNA-terminal protein circular complex and separation of the fragments by gel electrophoresis (Robinson *et al.*, 1973). It was therefore possible to establish that a 5.7×10^6 m.wt. fragment from one end of CELO virus DNA was missing in the hepatoma cell line that was T antigen negative, but was present in the two cell lines that were T antigen positive. Possible implications of these results will be discussed in the following chapter.

A number of lines of evidence suggest that viral DNA is covalently linked to, i.e., integrated into, cellular DNA in adenovirus transformed cells. The first indirect evidence for this was the reported isolation from Ad2 and Ad7 infected cells of RNA molecules which contained covalently joined viral and cellular RNA sequences (Tseui *et al.*, 1972; Wall *et al.*, 1973). It was suggested that these RNA molecules might have arisen from continuous transcription of integrated viral and contiguous cell DNA sequences. Bellett (1975) and Green *et al.* (1976) reported the covalent integration of adenovirus DNA in hamster cells transformed by CELO virus and Ad12 respectively. In both cases the network technique (Varmus *et al.*, 1973) was used and it appeared that all the viral DNA in the transformed cells was present exclusively in an integrated form.

If the integration of viral DNA into cellular DNA is a result of early virus-cell interactions, this can best be investigated during abortive infection by adenovirus of cells susceptible to transformation. Hamster (BHK) cells abortively infected by Ad12 have been extensively characterised (Strohl *et al.*, 1968; Strohl, 1969a; 1969b). Doerfler (1968) investigated the fate of Ad12 DNA in BHK21 cells at various times after infection with ^{3}H-thymidine (dThd) labelled Ad12 virus. Prior to

infection, the cells had been grown in the presence of BUdR in order to increase the density of the cellular DNA. The fate of the viral DNA could then be monitored by the distribution of ^{3}H label between the 'heavy' cellular and the 'light' viral peaks in CsCl density gradients. At early times after infection all the ^{3}H label has the density of virion Ad12 DNA. At late times (from 16 h post infection onward) an increasing amount of ^{3}H label was found associated with 'heavy' cellular DNA both in neutral and alkaline CsCl. Upon sonication the label shifted to a density position intermediate between those of cellular and viral DNA. These results were interpreted as evidence for covalent integration between viral and cellular DNAs.

However, these experiments did not rigorously exclude the possibility that tritiated degradation products of viral DNA could have been incorporated into cellular DNA and be thus misinterpreted as integrated viral DNA sequences. That this was indeed the case was demonstrated by the experiments of zur Hausen and Sokol (1969). Their results demonstrated that in Nil-2 hamster cells infected with ^{3}H-dThd - Ad12 virus, a substantial proportion of the input virus is degraded within 24 h and that ^{3}H-labelled degradation products are incorporated into replicating cellular DNA. However, they did not exclude the possibility that a fraction of the ^{3}H counts associated with the cell DNA truly represented integrated ^{3}H-Ad12 DNA sequences.

Further experiments were therefore necessary to assess the nature of the association of ^{3}H label with cellular DNA in hamster cells infected with ^{3}H-Ad12 virus (Doerfler, 1970). To avoid the incorporation of degradation products of ^{3}H-Ad12 DNA into replicating cellular DNA, DNA synthesis was inhibited by the addition of cytosine arabinoside from 2 h before to 44 h after infection. The inhibition of cellular DNA synthesis did not impair the association of ^{3}H label with cellular DNA fractionated

on alkaline CsCl density gradients. In addition, the ^{3}H label in cellular DNA hybridised predominantly with viral DNA and to a lesser extent with cellular DNA. These experiments provided some evidence that Ad12 DNA integrates, although some degradation and incorporation of viral nucleotides into cellular DNA might occur. This would affect quantitation of the amount of viral DNA integrated calculated from the specific activity of the DNA of ^{3}H-Ad12 input virus. It was calculated in this way that, 28 to 53 h post infection, 20 to 444 genome equivalents of Ad12 DNA per cell persisted, of which as many as 67 percent were integrated into cellular DNA. At later times in infection, the fraction of viral DNA linked to cellular DNA increased.

However, later experiments on the same system (Doerfler and Fanning, 1976) corrected the values reported above of the viral DNA persisting in hamster cells abortively infected by Ad12. The amount of persisting viral DNA was quantitated by DNA-DNA reannealing kinetics. It appeared to be independent of the multiplicity of infection used and to decrease steadily at times after infection until it stabilised after 48 h at approximately four genome equivalents of viral DNA per cell. This amount is far lower than that previously estimated (Doerfler, 1970). These results are also in contradiction with the previous ones which suggested that more viral DNA was integrated at late times in Ad12 abortively infected hamster cells. It appears therefore that the increased amounts of ^{3}H label associated with cell DNA were due to incorporation into replicating cellular DNA of ^{3}H products derived from degradation of ^{3}H-labelled virus.

The finding that integration of adenovirus DNA occurs in abortively infected cells and that viral DNA sequences are integrated in transformed cells, raised the possibility that integration might be an early event in any infection with adenovirus, independent of the ultimate outcome of the infection. Integration during productive infection by adenovirus would

provide a convenient system for studying the mechanics of integration. The molecular events and genetic functions of adenoviruses are well enough characterised during lytic infection to allow an investigation of which viral function(s), if any, are required for integration.

On the other hand, investigations on the integration of adenovirus DNA in productively infected cells are complicated, as in the case of the papovaviruses, by the presence within the cells of large amounts of newly replicated viral DNA. This is difficult to remove completely from any preparation of infected cell DNA before assaying for integrated viral DNA, and the contamination with free viral DNA must be estimated. Fortunately, high molecular weight, intracellular replicative forms of viral DNA, like those described for SV40, do not seem to occur during adenovirus DNA replication (Horwitz, 1971; Bellett and Younghusband 1972; Sussenbach and van der Vliet, 1972; Pearson, 1975; Stillman and Bellett, 1977) and are therefore most unlikely to contaminate cellular DNA.

Investigations on the intracellular forms of viral DNA in human KB cells productively infected with Ad2 led to the detection of at least four size classes of newly synthesised DNA sedimenting in alkaline sucrose at >100S, 50-90S, 34S (viral) and <20S (Burlingham and Doerfler, 1971; Burger and Doerfler, 1974; Doerfler *et al.*, 1974; Schick *et al.*, 1976; Fanning and Doerfler, 1977). These DNA species were detected by labelling Ad2 infected cells with ^{3}H-thymidine (^{3}H-dThd) for two or more hours. They all appeared to contain some viral DNA sequences, as judged by hybridisation experiments. The 50-90S DNA species showed properties intermediate between those of cellular and viral DNA (Doerfler *et al.*, 1974; Schick *et al.*, 1976). Thus it is likely that some viral DNA is integrated in cellular DNA during lytic infection. From 2000 to 7000 copies of Ad2 DNA per cell were detected at late times of infection associated with the 50-90S DNA (Doerfler *et al.*, 1974). The number of

copies of viral DNA integrated per cell might have been overestimated, since no account was taken for contaminating free viral DNA. Indeed, when the 50-90S DNA was cleaved with restriction endonucleases, viral DNA fragments identical to those generated by restriction endonuclease cleavage of virion DNA were obtained, together with viral DNA fragments of new sizes presumably derived from covalently linked cell and viral DNA. Most likely some contaminating free viral DNA produced the fragments identical to those produced by restriction cleavage of virion Ad2 DNA. On the whole, the restriction endonuclease digestion pattern of the viral DNA suggested that Ad2 DNA may be integrated in fragments rather than as in intact molecules in the cellular DNA (Baczko *et al.*, 1978).

These experiments, though providing some evidence that integration of viral DNA occurs during productive infection, are subject to two types of criticisms: (1) The labelled high molecular weight DNA may be mostly cellular. This is supported by the fact that no one has detected pulse-labelled replicative adenovirus DNA sedimenting faster than virion DNA in alkaline sucrose gradients (Horwitz, 1971; Pearson and Hanawalt, 1971; Sussenbach and van der Vliet, 1972; Bellett and Younghusband, 1972; Pettersson, 1973; Pearson, 1975; Stillman and Bellett, 1977). The 50-90S and 100S DNA species were also found in uninfected cells, and hybridised extensively to cellular DNA; and (2) the experiments cannot provide a reliable quantitative estimate of the integrated viral DNA using reannealing kinetics, since the amount of contamination by free viral DNA was not measured and allowed for. Thus, there is some evidence that integration occurs in productively infected cells as well as in cells abortively infected by adenovirus, and it appears to be a fairly common event. However, the present data do not allow any further conclusions.

Viral or cellular DNA replication are not prerequisites for integration (Doerfler, 1970). Integration of viral DNA is very unlikely to

be a prerequisite for viral DNA replication since more viral DNA is integrated at late times in lytically infected cells (Doerfler *et al.*, 1974; Fanning and Doerfler, 1976). This fact suggests also that the increased amount of integrated viral DNA at late times in infection, when large quantities of free viral DNA are present, might be due to an increased chance of recombination between viral and cellular DNA. Again it can be suggested, as it was for the papovaviruses, that this extensive integration of viral DNA into cellular DNA during lytic infection might contribute to cell death. It is interesting in this respect that infection of most species of cells with adenoviruses causes extensive chromosomal damage to the cell, leading to eventual cell death (Stich and Yohn, 1967; 1970; Strohl, 1969a; 1969b; 1973; zur Hausen, 1973). Random and extensive integration of the viral DNA into the cellular chromosome might be responsible for the irreversible chromosomal aberrations in the host cell.

CONCLUSIONS

The biological significance of the integration of viral DNA into the host cell chromosome is at present practically unknown. The best understood case of integration of viral DNA is represented by the lysogenic bacteriophages. These code for gene products which are required for integration, which occurs in some cases by a highly specific recombinational system. In the best described case of λ, an equally efficient system for excision of the integrated viral DNA is also encoded in the λ genome. This seems to suggest that integration has a definite role in the life cycle of the virus. However, integration does not conform to the same model in all bacteriophages. Mu differs from λ with respect to the lack of specificity in the sites of integration on the bacterial chromosome and to an increased rate of integration following prophage induction. In other words,

integration of Mu DNA and lytic replication of Mu are not mutually exclusive, as they are with λ. The presence of cellular DNA sequences at the ends of Mu DNA has indeed suggested that virion DNA arises from excision of viral DNA from covalently joined cellular DNAs.

The significance of integration during lysogeny is a function of the significance of lysogeny itself. This can be viewed as the result of an evolutionary relationship between phages and bacteria, since it depends, in most cases, on homology between regions of viral and cellular DNA. Probably lysogeny assumes significance in relation to its survival value for the virus. Integration of the viral DNA in the cellular DNA and the subsequent synchronous replication of viral and cellular genomes represent an extreme form of parasitic adaptation. As previously discussed, lysogeny via integration, adds new genetic information to the bacterial chromosome and can induce stable and inheritable changes in the lysogenised cell. These properties of the lysogenised cells can be compared to those of cells transformed by animal viruses.

Among animal viruses, integration of viral DNA in RNA tumour virus infected cells appears to have distinct functions, different from those exerted by integration of viral DNA in DNA tumour virus infected cells. This stems from essential differences in the nature and biology of the two types of viruses. In RNA tumour virus infected cells, integration of the proviral DNA is an essential prerequisite for virus replication and cell transformation, which can occur concomitantly in the same cell. RNA tumour viruses can therefore induce tumours in their natural hosts, or transform cells derived from their natural hosts without killing such cells.

Integration of the DNA provirus into the host cell chromosome can account for the stable inheritance of the transformed state and the genetic transmission of RNA tumour viruses. It is possible that a specific viral coded integration mechanism is necessary to preserve the specific

functions of the integrated provirus. The data available suggests that the RNA tumour virus genome can circularise and integrates at a specific site on the proviral DNA.

It is difficult on the other hand to attribute any specific function to the integration of viral DNA in cells infected by most DNA tumour viruses. Integration occurs during both lytic and abortive infection and the DNA in transformed cells is integrated. It appears to be random, with no specificity for the sites of integration either on the host or the viral genome.

Integration *per se* appears thus to be the result of an active recombinational system in the infected cell. All DNA tumour viruses induce chromosomal aberrations and extensive damage to the cells they infect. These might at least in part be the result of extensive integration into the cellular DNA which could therefore contribute to death of the cell during lytic or abortive infection.

With regard to the relation between integration of viral DNA and virus induced cell transformation, there is no proof for a causal relationship between them, but only circumstantial evidence for their association. The isolation of revertants from cells transformed by DNA tumour viruses, which still contain integrated viral DNA sequences indicates that integration *per se* does not induce transformation. Integration of viral sequences in host regulatory genes may induce irreversible mutations in the host chromosome, as occurs with Mu. However, since viral functions appear to be necessary for the establishment and/or the maintenance of transformation, it may simply be that integration of the viral genes involved is necessary for their persistence and continuous expression in the transformed cell.

EXPERIMENTS AND RESULTS

Chapter 2

THE INTEGRATION OF CELO VIRUS DNA

DURING LYTIC INFECTION

INTRODUCTION

Chicken embryo lethal orphan (CELO) virus is an adenovirus often found in chicken eggs and causes an inapparent infection in chickens (Yates *et al.*, 1960). The virus grows productively and to high yields in primary cultures of chicken embryo kidney (CEK) cells (Younghusband and Bellett, 1971; Bellett and Younghusband, 1972). The genome of CELO virus is a linear molecule of double stranded DNA, with an average molecular weight of 28 x 10^6 (Laver *et al.*, 1971; Younghusband and Bellett, 1971; Robinson *et al.*, 1973). When extracted from purified virions by methods excluding the use of proteolytic enzymes, the CELO virus chromosome is released as a circular DNA protein complex (Robinson *et al.*, 1973). Circularisation of the molecule is achieved by a protein that links the ends of the DNA molecule as described for other adenoviruses (Robinson and Bellett, 1974; Rekosh *et al.*, 1977). The structure and the replication of CELO virus DNA have been extensively investigated (Younghusband and Bellett, 1971; Bellett and Younghusband, 1972). Mature CELO virus DNA consists of a unique rather than a permuted collection of linear molecules. These molecules lack both exposed single stranded complementary ends and duplex terminal repetitions. However, an inverted terminal repetition, about 110 nucleotides long, is at the ends of the duplex DNA molecule (Robinson and Bellett, 1975). All these features of the CELO virus DNA molecule are consistent with those described for human adenovirus serotypes (Garon *et al.*, 1972; Wolfson and Dressler, 1972; Robinson *et al.*, 1973).

The replication of CELO virus DNA is semiconservative and occurs via linear intermediates (Bellett and Younghusband, 1972). Replicating CELO virus DNA contains extensive single stranded regions. In alkaline sucrose

gradients the growing strands of the virus DNA sediment more slowly than those of mature DNA, and labelled parental DNA sediments with virion DNA. These results eliminate modes of replication involving continuous concatemers or the addition of new material to parental strands, as in the rolling circle model. No evidence for closed circular structures of CELO virus DNA has been found (Bellett and Younghusband, 1972). Similar results have been obtained in many laboratories with human adenoviruses (Horwitz, 1971; Pearson and Hanawalt, 1971; Sussenbach and van der Vliet, 1972; Pettersson, 1973; Pearson, 1975). These data cannot exclude the existence in adenovirus infected cells of concatemeric forms of replicating DNA containing single stranded interruptions. However, with our present knowledge of adenovirus DNA structure, it is difficult to envisage how these forms could be generated.

A genetic or physical map of CELO virus DNA is not yet available. Ishibashi and Ito (1971) isolated 49 temperature sensitive mutants of CELO virus which were assigned to five groups (not complementation groups) on the basis of the distribution of capsid antigens in the cell as detected by immunofluorescence. In this way mutants of CELO virus with anomalous transport of viral structural antigens could be detected. One mutant, ts22 from group V produced no viral antigens, and no viral DNA synthesis seemed to occur in the infected cell at the non-permissive temperature. Viral DNA synthesis was detected in cells infected with mutants from other groups. It is therefore possible that the ts22 mutant of CELO virus represents an 'early' DNA negative mutant.

These mutants could be useful for investigations of the CELO virus coded functions during infection and for the identification of the viral polypeptides. Very recently Yasue and Ishibashi (1977) reported that at least 23 polypeptides are induced by CELO virus in productively infected cells, eight of which are synthesised 'early' in the presence of AraC.

CELO virus is oncogenic. It induces tumours in hamsters (Sarma *et al.*, 1965; Mancini *et al.*, 1969; Mancini *et al.*, 1970; Stenbach *et al.*, 1973) and transforms human (Anderson *et al.*, 1969a) and hamster cells in culture (Anderson *et al.*, 1969b). CELO virus transformed hamster cell lines have also been derived from CELO virus induced hamster tumours (Stenback *et al.*, 1973). Like other adenovirus transformed cells, CELO virus transformed cells do not produce virus (Potter and Oxford, 1969; Schild *et al.*, 1970). The CELO virus specific T antigen is however detected in only some CELO virus induced tumour cells, and in *in vitro* established CELO virus transformed cell lines (Potter and Oxford, 1969; Jones *et al.*, 1970; Schild *et al.*, 1970; May *et al.*, 1978). CELO virus T antigen does not cross react with T antigens produced by adenoviruses of other species (Potter and Oxford, 1969; Schild *et al.*, 1970).

CELO virus transformed cells contain virus specific DNA sequences (Bellett, 1975). *In vitro* CELO virus transformed hamster skin (THS) cells contain DNA sequences homologous to most of the CELO virus DNA molecule. Investigations with EcoRI restriction endonuclease fragments of CELO virus DNA detected six of the seven fragments in these cells (May *et al.*, 1975). The missing fragment, of molecular weight (m. wt.) 2×10^6 represents one end of the CELO virus DNA molecule (Robinson *et al.*, 1973). The other EcoRI restriction fragments of CELO virus DNA were present in approximate equimolar amounts (3.5 equivalents per diploid amount of cell DNA), with the exception of the EcoRI A fragment, which was under-represented. The THS cells were T antigen positive.

Interestingly, a T antigen negative transformed cell line derived from a CELO virus induced hepatoma, lacks the viral DNA sequences homologous to the 5.7×10^6 m. wt. EcoRI B fragment of CELO virus DNA, which corresponds to the opposite end of the CELO virus DNA molecule to that missing in THS (T antigen +) cells (May *et al.*, 1978).

In another line of CELO virus induced hamster hepatoma cells, which are T antigen positive, copies of most of the viral genome were present (May *et al.*, 1978) including the terminal fragment present in THS cells (May *et al.*, 1975). These results suggest that the gene for the CELO virus T antigen lies within 20 percent (5.7×10^6) of one end of the DNA. However, since the T antigen negative hepatoma cell line is transformed and tumorigenic, integration and expression of this portion of the DNA is not always required to maintain the altered cell phenotype. This is consistent with the fact that most CELO virus induced tumours are T antigen negative (Jones *et al.*, 1970).

In the THS CELO virus transformed cells described above, the viral DNA sequences are integrated into the cellular DNA (Bellett, 1975). DNA-DNA reannealing kinetics with the whole CELO virus DNA as probe showed that viral sequences were covalently associated with 'networks' of cellular DNA (Varmus, 1973). An improvement in the original 'network' method allowed an exact quantitation of the integrated viral DNA. It was calculated that 2.5 equivalents of CELO virus DNA per transformed cell were integrated and these accounted for the total amount of viral DNA present in the transformed cell.

CELO virus therefore resembles the human oncogenic adenoviruses including its capacity to integrate its genome into the cellular chromosome. The presence of viral DNA sequences exclusively integrated in the host chromosome appears to be a stable property of adenovirus transformed cells (Bellett, 1975; Green *et al.*, 1976). However, the fact that in cells abortively infected with adenovirus (Doerfler, 1968; 1970; Fanning and Doerfler, 1976) integrated viral DNA appears to be present in many copies per cell on average, while very few cells will become transformed, makes it unlikely that the frequency of integration alone determines the frequency of transformation. In this respect,

investigations designed to define which viral DNA sequences are integrated and how specific are the sites of recombination on both cellular and viral genomes should be informative. Another approach is to find out whether integration is a general phenomenon in adenovirus infected cells, which occurs in any type of infection irrespective of the outcome of the infection itself.

In this chapter, experiments on the integration of CELO virus DNA in productively infected CELO virus cells will be reported. The integration of human Ad2 DNA in productively infected KB cells has been studied by others (Burger and Doerfler, 1974; Fanning and Doerfler, 1976; Schick *et al.*, 1976; Baczko *et al.*, 1978). As described previously, as many as 2000 to 7000 copies of viral DNA per cell were found by reassociation kinetics in a fast sedimenting zone (50S to 90S) in alkaline sucrose gradients after ^{3}H-dThd labelling of Ad2 infected KB cells (Burger and Doerfler, 1974; Fanning and Doerfler, 1976; Schick *et al.*, 1976). This high molecular weight DNA form appeared very early in infection when newly synthesised virion size Ad2 DNA (34S) was not yet detected. As early as 5 h post infection 1000-3000 copies per cell of viral DNA were associated with the 50-90S DNA. Radioactivity associated with this zone appears to be mainly due to replicating cellular DNA since it sediments more slowly than the bulk of cell DNA, but appears in the main cell DNA peak after a 'chase'. It is also found in uninfected cells, its rate of reassociation is increased by cellular DNA, and it reassociates with kinetics similar to those of eukaryotic DNA. However, this radioactive DNA has a density between that of viral and cellular DNA and contains some sequences that hybridise with viral DNA (Doerfler *et al.*, 1974; Schick *et al.*, 1976; Baczko *et al.*, 1978). This DNA species is not due to fast sedimenting viral DNA replicative intermediates (Horwitz, 1971; Pearson and Hanawalt, 1971; Bellett and Younghusband, 1972; Sussenbach

and van der Vliet, 1972; Pearson, 1975; Pettersson, 1976, Stillman and Bellett, 1977). It seems likely that some of the viral DNA detected in this zone by reassociation kinetics is integrated, since its restriction pattern apparently differs from that of virion DNA (Schick *et al.*, 1976; Baczko *et al.*, 1978). Thus these reports provide some evidence for integration of viral DNA during lytic infection.

However, the large amount of free progeny viral DNA is difficult to remove completely from any preparation of infected cell DNA before assaying for integrated viral DNA, and the remaining contamination with free viral DNA should have been estimated. Such experiments can also be complicated by the possible existence of forms of replicating viral DNA that sediments with cell DNA in alkaline sucrose gradients. Fortunately these do not seem to occur in CELO virus or other adenovirus infections (Horwitz, 1971; Pearson and Hanawalt, 1971; Bellett and Younghusband, 1972; Sussenbach and van der Vliet, 1972; Pearson, 1975; Pettersson, 1976; Stillman *et al.*, 1977). In a previous study of CELO virus DNA replication in productively infected CEK cells, no labelled parental viral DNA could be detected associated with cell DNA nor did any newly synthesised viral DNA (labelled in a pulse of 1 h or less) sediment faster than free viral DNA in alkaline sucrose gradients as in the Ad2 infected KB cells (Bellett and Younghusband, 1972). Other more sensitive methods for detecting the presence of integrated CELO virus DNA in the DNA of productively infected CEK cells were therefore developed and are described in the following results section.

EXPERIMENTS AND RESULTS

THE INTEGRATION OF CELO VIRUS DNA DURING LYTIC INFECTION

CELO virus replicates in primary CEK cells cultures and produces large amounts of intracellular viral DNA from 16 to 40 h post infection (Younghusband and Bellett, 1971). If integration is a general recombinational event in the adenovirus infected cell, it will occur more often and therefore be more easily detected when large amounts of viral DNA are present. For this reason a late time in infection (36 h) was first chosen for the present investigation. Of course, the large quantity of intracellular viral DNA present at that time post infection has to be removed from the preparation of infected cell DNA before assaying for integrated viral DNA, and the remaining contamination with free viral DNA must be estimated. Three different methods were used to separate the cell DNA from the free viral DNA. These depend on several differences between the viral and cellular DNA molecules, such as molecular weight, buoyant density, and sequence reiteration. The use of these three methods also overcomes the problem of the possible existence of yet undetected forms of free viral DNA behaving differently from the virion DNA. Cell DNA purified by these methods was tested for viral DNA sequences by reannealing kinetics. In each case uninfected cells plus a known amount of free viral DNA were used as a control.

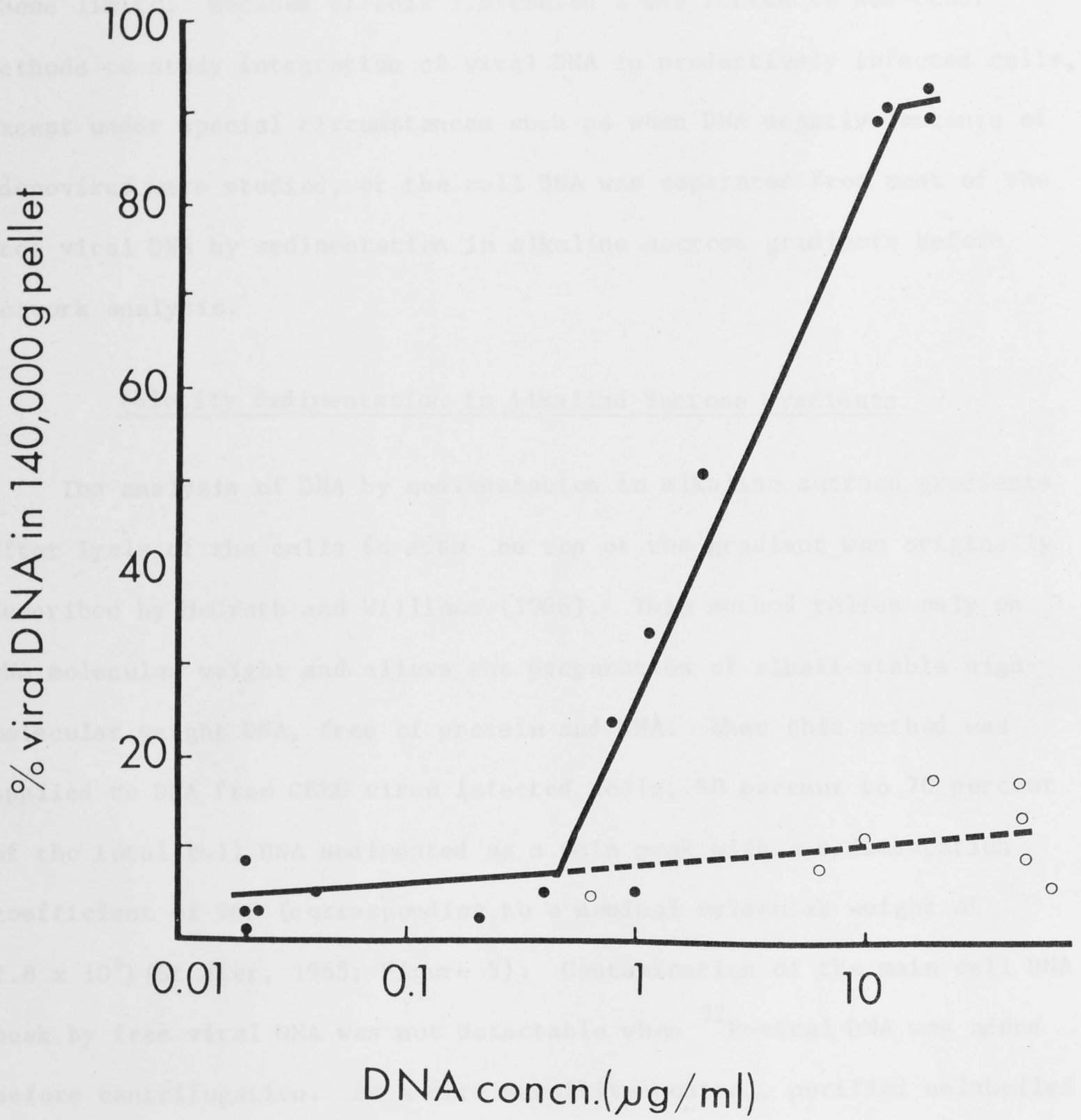
Limitations of the Network Technique

The network method (Varmus *et al.*, 1973) gives more conclusive evidence than most other methods for integration of viral DNA, because it depends on repeated sequences in cell DNA as well as on the difference in molecular weight between cell and viral DNA. The method has been used to demonstrate covalent integration of adenovirus DNA in transformed cells

(Bellett, 1975; Green *et al.*, 1976). However when network analysis of integration of viral DNA was attempted using total DNA prepared from cells late in productive infection, pelleting of free viral DNA with the cell DNA network occurred. This was probably due to aggregation of viral DNA by imperfect annealing at high concentration. To investigate the conditions under which this artefact occurred ^{32}P-CELO virus DNA was denatured when mixed with either unlabelled CELO virus DNA or calf thymus DNA at various concentrations, annealed for 2 h at 68°C in 0.6 M NaCl (the usual conditions for network formation), centrifuged at 140,000g for 15 min, and the ^{32}P in the network and supernatant fractions was counted. When ^{32}P-CELO virus DNA alone (30 ng/ml) was treated in this way, about 5 percent of the ^{32}P was pelleted. This increased gradually with increasing concentrations of calf thymus DNA to about 15 percent of the ^{32}P in the network at 80 µg/ml calf thymus DNA (Figure 4), while 60 percent to 70 percent of the calf thymus DNA was in the network pellet based on optical density measurements. This distribution of viral and cellular DNA was similar to that found previously with mixtures of CELO virus DNA and BHK cell DNA (Bellett, 1975). However, when unlabelled viral DNA was added to ^{32}P-viral DNA to give concentrations above 1 µg/ml, the amount of ^{32}P that sedimented into the network pellet increased dramatically to reach about 90 percent at 10 µg/ml (Figure 4). This did not occur when the same concentrations of native DNA or denatured DNA were added to the sample without annealing. To test whether the effect was due to annealing viral DNA at high concentration or to annealing to high Cot values, ^{32}P-CELO virus DNA was treated by the network procedure as above with 11 µg/ml unlabelled viral DNA and 80 percent of the ^{32}P pelleted. However, when the concentration of viral DNA was reduced tenfold and the annealing time increased tenfold to give the same Cot value, only 18 percent of the ^{32}P pelleted, compared with a background value of 5 percent. This suggests that the artefact is mainly due to annealing viral DNA at high concentrations.

FIGURE 4: Contamination of DNA network pellets with viral DNA

Contamination of the network pellet with ^{32}P-CELO virus DNA (30 ng/ml) was measured after denaturation, annealing for 2 h at 68°C and centrifugation at 140,000 g for 15 min in the presence of increasing concentrations of unlabelled CELO virus DNA (●——●) or calf thymus DNA (o---o). Results shown combine those from four separate experiments.


100
80
60
40
20
% viral DNA in 140,000 g pellet
0.01
0.1
1
10
DNA concn.(μg/ml)

The data in Figure 4 suggests that the network method is reliable below 0.1 µg/ml of free viral DNA and possibly up to 0.4 µg/ml. Previous experiments with transformed cell DNA (Bellett, 1975) were well within these limits. Because of this limitation I was forced to use other methods to study integration of viral DNA in productively infected cells, except under special circumstances such as when DNA negative mutants of adenovirus were studied, or the cell DNA was separated from most of the free viral DNA by sedimentation in alkaline sucrose gradients before network analysis.

Velocity Sedimentation in Alkaline Sucrose Gradients

The analysis of DNA by sedimentation in alkaline sucrose gradients after lysis of the cells *in situ* on top of the gradient was originally described by McGrath and Williams (1966). This method relies only on DNA molecular weight and allows the preparation of alkali-stable high-molecular weight DNA, free of protein and RNA. When this method was applied to DNA from CELO virus infected cells, 50 percent to 70 percent of the total cell DNA sedimented as a main peak with a sedimentation coefficient of 96S (corresponding to a nominal molecular weight of 2.8×10^8) (Studier, 1965; Figure 5). Contamination of the main cell DNA peak by free viral DNA was not detectable when ^{32}P-viral DNA was added before centrifugation. As a more sensitive control, purified unlabelled CELO virus DNA was added to uninfected cells before lysis in an amount equal to, or greater than, the amount of viral DNA expected in infected cells.

The gradients from either infected or uninfected cells were sub-divided into pools, and the amount of viral DNA in each pool was estimated by reannealing kinetics (Figure 6 and Table 1). Viral DNA corresponding to 905 copies per diploid amount of cell DNA was found specifically

FIGURE 5: Alkaline sucrose gradients of CEK cell DNA from (A) infected and (B) uninfected cells

Sedimentation is from right to left. CEK cells were prelabelled with ^{3}H-dThd prior to infection; (●---●): ^{3}H-cell DNA; (■---■): ^{32}P-CELO virus DNA added as sedimentation marker.

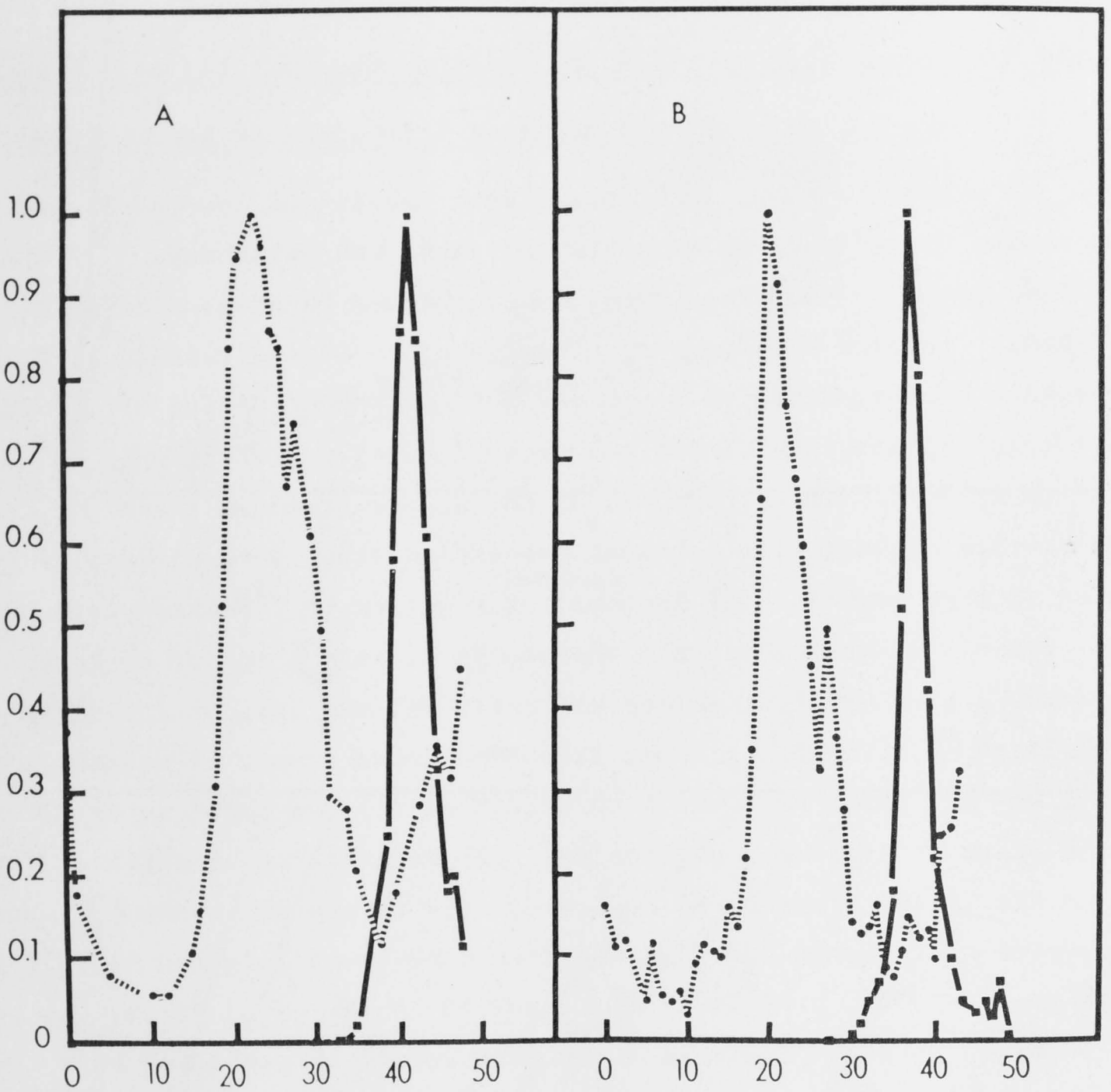
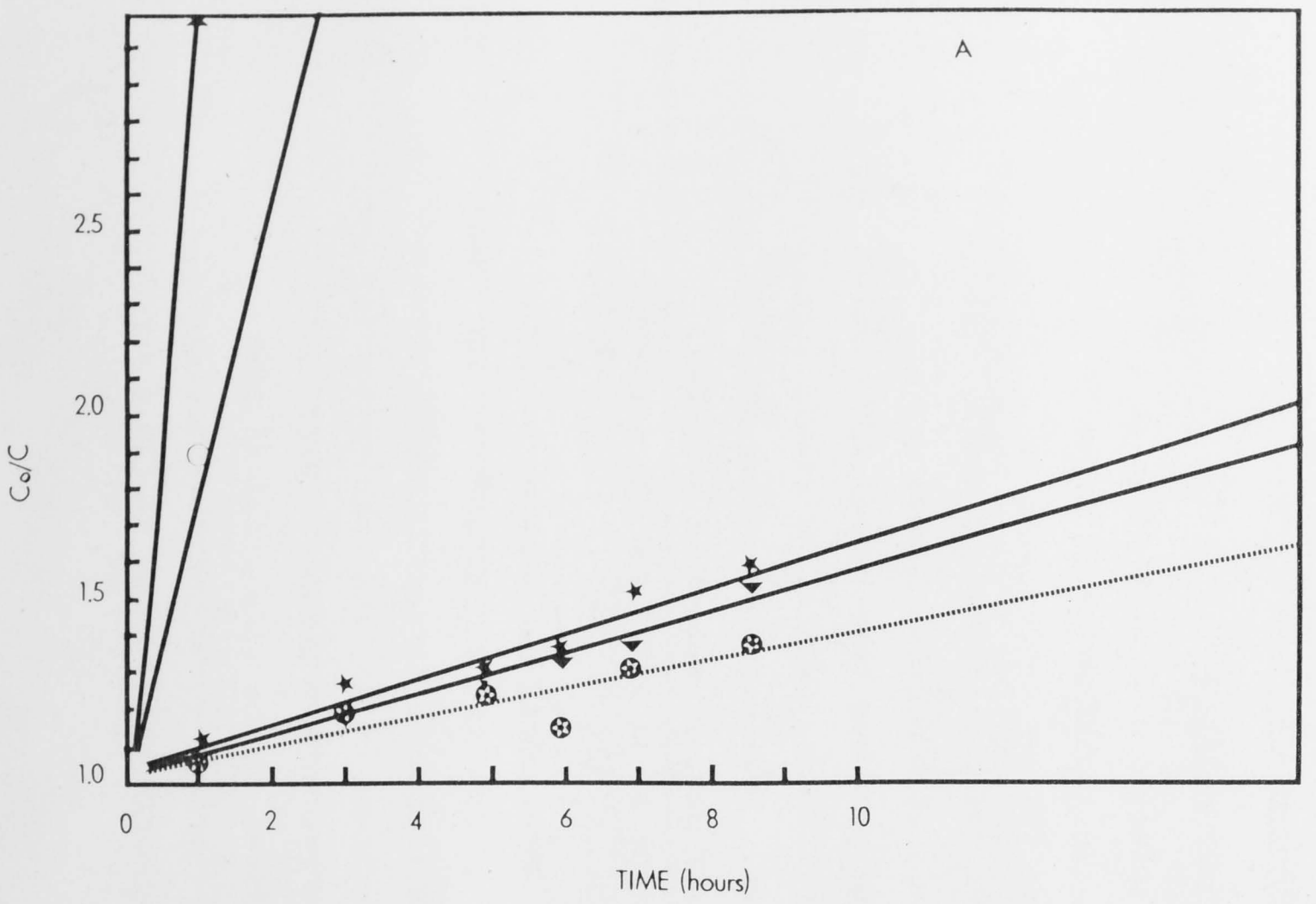

A
B
CPM RELATIVE TO PEAK
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
0
10
20
30
40
50
FRACTION NUMBER

FIGURE 6: Reannealing kinetics of ^{32}P-CELO virus DNA (17 ng/ml) in the presence of cell DNA isolated from alkaline sucrose gradients similar to those

The gradients, shown in Figure 5, were subdivided into pools and each was tested by reannealing kinetics for viral DNA sequences. ^{32}P-CELO DNA plus DNA from each pool was sonicated, denatured and reannealed at 68°C in 1 M NaCl. Samples were removed at intervals, diluted tenfold in 0.14 M phosphate buffer pH 6.8, 0.4 percent SDS, and the fraction of ^{32}P-DNA that remained single-stranded was determined by chromatography on hydroxylapatite at 60°C. The reciprocal of the single-stranded fraction, Co/C, was plotted against time. Linear regression lines were calculated by the least squares method. (A) Reannealing kinetics of ^{32}P-CELO virus DNA in the presence of sonicated calf thymus DNA alone ✪ and of sonicated calf thymus DNA plus DNA from uninfected cells (Figure 5B); pool 1-13 ▼; pool 14-28 ★; 29-38 ◯; pool 39-46 ◓. A known amount of unlabelled CELO virus DNA (≥4 µg/60 mm petri dish of CEK cells) was added to uninfected cells prior to lysis and processing. (B) Reannealing kinetics of ^{32}P-CELO virus DNA in the presence of sonicated calf thymus DNA alone ✪, and of sonicated calf thymus DNA plus DNA from CELO virus infected cells (Figure 5A); pool 1-13 ▼; pool 14-22 ☆; pool 23-28 ★; pool 29-38 ◯; pool 39-46 ◓. In each reannealing the same amount of sonicated calf thymus DNA was also present.


A
2.5
2.0
1.5
1.0
C_o/C
0
2
4
6
8
10
TIME (hours)

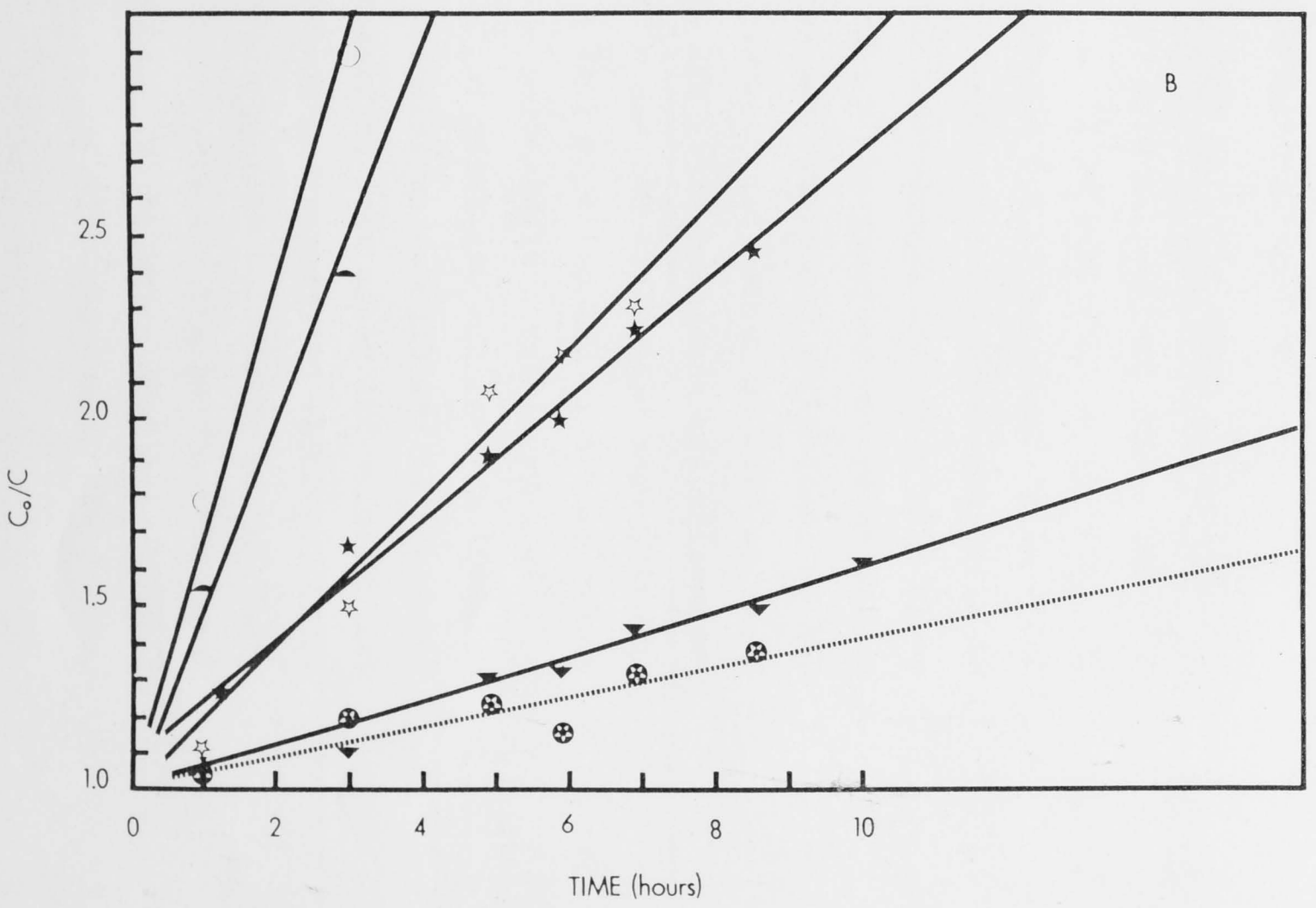

B
2.5
2.0
1.5
1.0
C_o/C
0
2
4
6
8
10
TIME (hours)

TABLE 1: Quantitative analysis of viral DNA associated with cell DNA after alkaline sucrose gradient sedimentation

CEK cells, prelabelled with ^{3}H-dThd, were infected with CELO virus at a multiplicity of infection of 22 to 23 i.v./cell and incubated at 36°C for 36 h. Cells were then lysed in 0.3 N NaOH - 0.5 percent SDS and centrifuged on alkaline sucrose gradients. The fraction numbers refer to gradients similar to those shown in Figures 5 and 6. DNA pools were processed as described and the recovery of DNA in each pool was monitored by the recovery of ^{3}H counts. Each DNA pool was then tested for viral DNA by reannealing kinetics: the amounts of viral DNA calculated by reannealing kinetics were corrected for recovery of ^{3}H counts. The number of copies per cell represents the number of viral DNA equivalents per diploid amount of cell DNA, assuming that CELO virus DNA has a molecular weight of 29×10^{6} and the diploid amount of DNA in CEK cells is 2.45×10^{-6} µg.

As control for contamination of cell DNA by free viral DNA a known amount of unlabelled viral DNA was added to ^{3}H-labelled, uninfected CEK cells which were then processed as the CELO virus infected cells. The fractions corresponding to viral and cellular DNA peaks were collected as with infected cells, and then tested for viral sequences by reannealing kinetics. In the table, the amount of viral DNA found in the uninfected cell gradient was normalised to the same total as that from infected cells for a direct comparison. Integrated viral DNA = (fractions 1 to 28 infected) - (fractions 1 to 28 control, uninfected) = 1.07 µg (19.4 percent) = 905 copies per cell.

For method of calculation of viral DNA in copies per cell and example see p126a

TABLE 1

QUANTITATIVE ANALYSIS OF VIRAL DNA ASSOCIATED WITH CELL DNA AFTER ALKALINE SUCROSE GRADIENT SEDIMENTATION

FRACTIONS (BOTTOM TO TOP)	CELL DNA (%)	µG OF VIRAL DNA		
		UNINFECTED CELLS PLUS VIRAL DNA	INFECTED CELLS AT 36 H POST INFECTION	
1-13	15.1	0.06	0.16	
14-22	45.8	0.09	0.66	1.06
23-28			0.40	
29-38	25.1	2.12	3.20	
39-46	14.0	3.24	1.09	

associated with the cell DNA from infected cells. This represents 19.4 percent of the total viral DNA after correction for contamination by free viral DNA, which was only 2.4 percent in the control experiment. This control accounts only for contamination by mature free viral DNA. However, like other adenoviruses, CELO virus DNA has inverted terminal repeated sequences, without cohesive ends or terminal direct repeats that would allow formation of concatemers or circles (Younghusband and Bellett, 1971; Robinson and Bellett, 1975). No replicative forms of CELO DNA that sediment faster than the parental strands could be detected in pulse-labelled DNA from infected CEK cells (Bellett and Younghusband, 1972). Recent experiments on the replication of adenovirus DNA have failed to find cross-linked replicative intermediates expected from a hairpin primed mechanism of replication of the 5' ends of adenovirus DNA (Stillman *et al.*, 1977). It is therefore unlikely that the viral DNA found associated with cell DNA by sedimentation in alkaline sucrose represents an undetected form of free viral DNA.

Network Technique

In order to test the reliability of sedimentation in alkaline sucrose gradients as a method of separation of cell DNA and integrated viral DNA from free viral DNA, we further analysed DNA prepared from infected cells on alkaline sucrose gradients by the network technique. However, 60-90 percent of added ^{32}P-viral DNA pelleted with the network fraction of the main cell DNA peak from alkaline sucrose gradients of infected cells, compared with 20 percent in the presence of DNA from uninfected cells. This could be due to annealing of ^{32}P-viral DNA to integrated viral DNA, to the artefact caused by high concentration of total viral DNA described above, or both. The total concentration of

TABLE 2: The effect of network purification on the distribution of viral DNA from alkaline sucrose gradients

CEK cells, prelabelled with ^{3}H-dThd, were infected with 1 i.u. of CELO virus per cell and incubated at 37°C for 36 h. Cells were then lysed, centrifuged on alkaline sucrose gradients, and processed as described. ^{3}H-DNA from the cell peak was tested by reannealing kinetics for viral DNA and has been corrected for contamination with free viral DNA found in the control gradient after normalization. The ^{3}H-DNA from the infected cell peak was then analyzed by the network method.

Network and supernatant fractions were assayed for viral DNA by reannealing kinetics, and the amount of integrated viral DNA was calculated as described in the text. Copies per cell represent viral DNA equivalents per diploid amount of cell DNA.

For method of calculation of viral DNA in copies per cell and example see p126a

TABLE 2

THE EFFECT OF NETWORK PURIFICATION ON THE DISTRIBUTION OF VIRAL DNA FROM ALKALINE SUCROSE GRADIENTS.

SAMPLE	AMOUNT OF VIRAL DNA		
	VIRAL PEAK	CELL PEAK	
	(μG)	(μG)	(COPIES/CELL)
UNINFECTED CELLS	5.88	0.16	
INFECTED CELLS (36H P.I.)	5.6	0.44	904
INFECTED CELLS CORRECTED VALUE	5.8	0.28	580
NETWORK PURIFICATION OF CELL PEAK FROM INFECTED CELLS.		0.243	503

viral DNA was calculated to be still more than 1 µg/ml in these experiments, which is outside the limits within which the method is reliable. To overcome this problem a very small amount of ^{3}H-labelled infected cell DNA (from alkaline sucrose gradients) was diluted with DNA from uninfected cells in order to have total amounts of both viral and cell DNA suitable for network analysis. ^{3}H-labelled DNA from infected cells was added at a final concentration of 6 to 8 µg/ml (70 ng of viral DNA per ml) to about 300 µg of DNA per ml extracted from uninfected cells.

After sedimentation in alkaline sucrose and correction for contamination by free viral DNA, 0.28 µg of viral DNA was found associated with cell DNA from 7 x 10^{6} cells. This DNA was then analysed by the network method. Most (82 percent) of the ^{3}H-labelled cell DNA and 36 percent of the added free ^{32}P-labelled CELO virus DNA sedimented with the unlabelled cell DNA network after denaturation and annealing. The network and supernatant fractions were then tested for viral DNA sequences, and the results were analysed quantitatively using an equation that corrects for shearing of cell DNA and for trapping of free viral DNA in the networks (Bellett, 1975). Of the viral DNA (0.28 µg) originally associated with cell DNA in alkaline sucrose, 87 percent (0.24 µg) was found to be specifically associated with the cell DNA by network analysis (Table 2). This suggests that the viral DNA associated with cell DNA in alkaline sucrose is integrated.

CsCl Buoyant Density Gradients

To further exclude the presence of as yet undetected concatemeric or circular replicating forms of viral DNA that could sediment with cell DNA in alkaline sucrose gradients, the cell DNA was separated from the viral DNA by buoyant density sedimentation. The cell DNA was prelabelled with 2-^{3}H-adenosine and BUdR to give a better separation of free viral

FIGURE 7: Neutral and alkaline CsCl gradients of the total intracellular DNA from CELO virus infected cells and from uninfected cells

CEK cells were prelabelled with BUdR and [^{3}H]2-Adenosine. At the time of infection the cells were thoroughly washed to remove ^{3}H-Adenosine and BUdR and fresh medium containing 10^{-5}M thymidine was added. The cells were lysed at 36 h p.i., total intracellular DNA was extracted and centrifuged on CsCl gradients. A known amount (see Table 3) of unlabelled CELO virus DNA was added to the uninfected CEK cells prior to lysis. ^{3}H adenosine ▲ - - - - ▲, ^{32}P viral DNA ● ——— ●

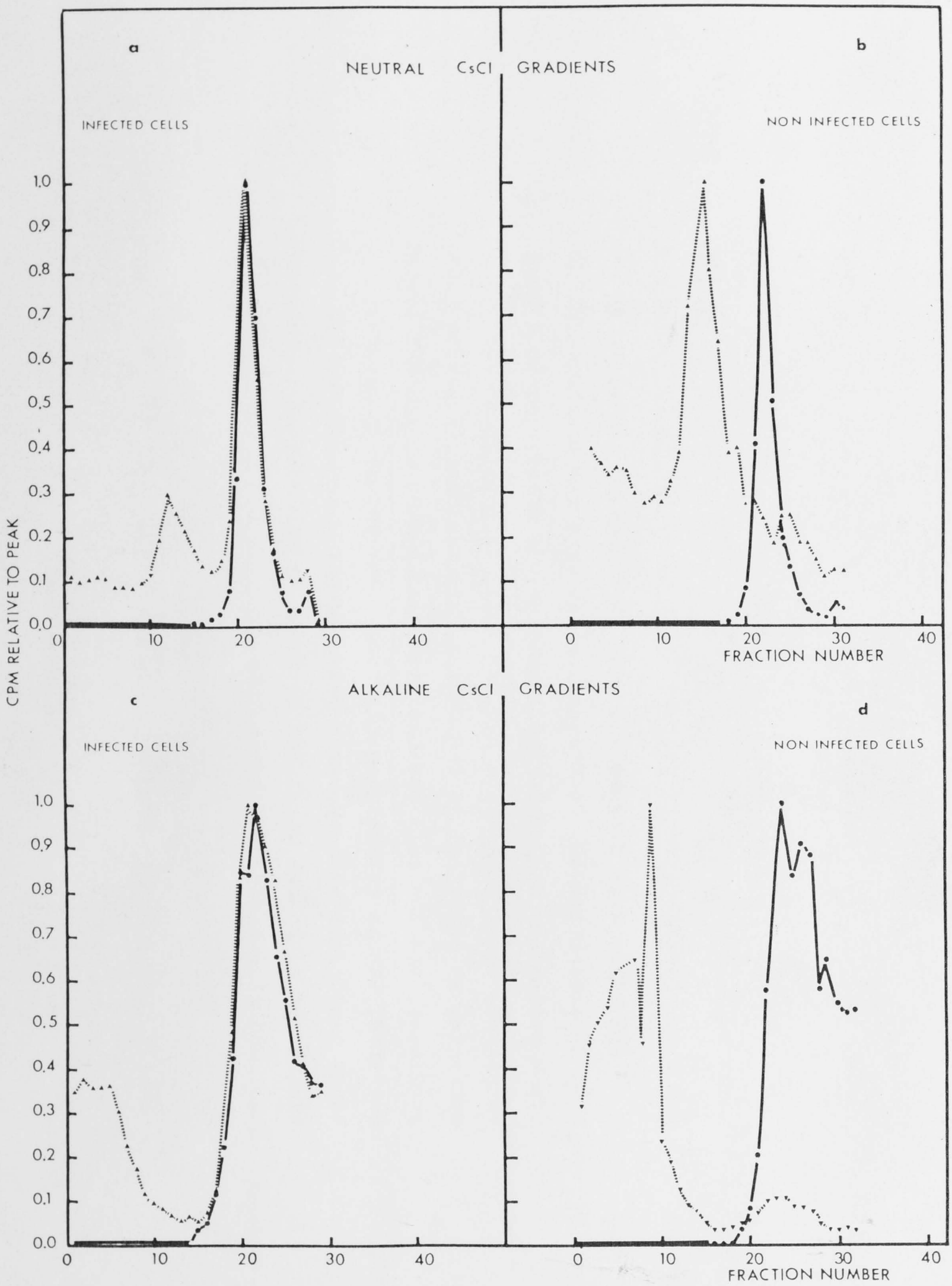

a
NEUTRAL CsCl GRADIENTS
INFECTED CELLS
b
NON INFECTED CELLS
CPM RELATIVE TO PEAK
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
FRACTION NUMBER
ALKALINE CsCl GRADIENTS
c
INFECTED CELLS
d
NON INFECTED CELLS

TABLE 3: Distribution of intracellular viral DNA in CsCl gradients

CEK cells were prelabelled with ^{3}H-adenosine and BUdR; at the time of infection with CELO virus, cells were washed thoroughly and fresh medium containing 10^{-5} M thymidine was added. At 36 h post infection, cells were lysed and the total intracellular DNA was extracted as described in the text. This was centrifuged on CsCl gradients, and the cell and the viral peaks were pooled and tested for viral DNA by reannealing kinetics. The amount of viral DNA found associated with the infected-cell DNA was corrected for contamination with free viral DNA as found in the control (uninfected-cell DNA plus added CELO virus DNA).

For method of calculation of viral DNA in copies per cell and example see p126a

TABLE 3

DISTRIBUTION OF INTRACELLULAR VIRAL DNA IN CsCl GRADIENTS

SAMPLE	VIRAL DNA					
	NEUTRAL CsCl			ALKALINE CsCl		
	VIRAL PEAK (μG)	CELL PEAK		VIRAL PEAK (μG)	CELL PEAK	
		μG	COPIES/CELL		μG	COPIES/CELL
UNINFECTED CELLS PLUS VIRAL DNA	4.97	0		4.44	0.04	
INFECTED CELLS AT 36 H POST INFECTION	4.70	0.27		4.20	0.28	
INFECTED CELLS (CORRECTED VALUE)	4.70	0.27	993	4.24	0.24	1,087

DNA from cell DNA. The cell DNA had a density of between 1.87 and 1.84 g/ml (very close to the bottom of the gradient) in alkaline CsCl and between 1.77 and 1.76 g/ml in neutral CsCl (Figure 7).

The cells were washed and changed to normal medium without BUdR and containing 10^{-5}_{M} dThd at the time of infection. Incorporation of ^{3}H-labelled nucleotides into viral DNA occurred probably because all nucleotide pools were labelled during preincubation in ^{3}H-adenosine. However the labelled viral DNA was of light density because it sedimented together with the ^{32}P-labelled viral DNA marker of density 1.713 g/ml, showing that BUdR in cell DNA was not reutilised. The peak of ^{3}H-labelled virion DNA did not occur when ^{3}H-BUdR was used to prelabel the cell DNA. The distribution of viral DNA between the cell and the viral peaks was determined by reassociation kinetics. Again, uninfected cell DNA with added unlabelled CELO virus DNA was used as a control. Table 3 shows that only 0.8 percent of the CELO virus DNA added to the control contaminated the cell DNA in the alkaline CsCl gradients, and none could be detected in neutral CsCl. From 993 to 1,087 copies of viral DNA per cell were associated with the cell DNA after correction for contaminating free viral DNA. This value is very close to and confirms the result found by alkaline sedimentation (905 copies per cell) in previous experiments using a similar multiplicity of infection (Table 1).

Time Course of Integration of CELO virus DNA in Infected Cells

The time course of the integration of CELO virus DNA into CEK cell DNA was investigated in relation to the replication of CELO virus DNA. CELO virus-infected CEK cells were lysed and sedimented on alkaline sucrose gradients at different times post infection. As a zero time sample the CEK cells were allowed to adsorb the virus inoculum for 2 h and then lysed. Contamination of the cellular DNA by free viral DNA

TABLE 4: Time of integration of CELO virus DNA into CEK DNA

CELO virus infected CEK cells were lysed and sedimented on alkaline sucrose gradients at different times post infection. The cell and the viral peaks were each collected and tested by reannealing kinetics for viral DNA. As control for contamination of cell DNA by free viral DNA a known amount of unlabelled viral DNA was added to uninfected CEK cells which were then processed as CELO virus infected cells. The fractions corresponding to viral and cellular DNA peaks were collected as with infected cells, and then tested for viral sequences by reannealing kinetics. In the table, the amount of viral DNA found in the uninfected cells gradient was normalized to the same total as that from infected cells for a direct comparison. To estimate the amount of viral DNA integrated (c), viral DNA in the cell peak has been corrected for contamination with free viral DNA calculated from the control gradient after normalization.

For method of calculation of viral DNA in copies per cell and example see p126a

TABLE 4

TIME OF INTEGRATION OF CELO VIRUS DNA INTO CEK DNA

TIME (H P.I.)	TOTAL VIRAL DNA EQUIVALENTS	TOTAL VIRAL DNA SEDIMENTING WITH CELL DNA (FR 2-28) IN ALKALINE SUCROSE (COPIES/CELL)	INTEGRATED VIRAL[b] DNA (COPIES/CELL)
0	0.1×10^{10}	44	U.D[a]
10	0.4×10^{10}	U.D	U.D
18	0.8×10^{10}	343	279
24	2.1×10^{10}	706	646
36	7.0×10^{10}	1014	950
CONTROL	7.0×10^{10}	64	

a UNDETECTABLE

b INFECTED CELL VALUE - CONTROL

was again estimated by the addition of virion CELO virus DNA to uninfected CEK cells, which were then processed as were the CELO virus-infected cells. The cell and the viral DNA peaks from the alkaline sucrose gradients were then analysed by DNA-DNA reannealing kinetics to quantitate viral DNA.

Integration of CELO virus DNA into CEK DNA was not detected until 18 h post infection and its rate increased with the increase in the amount of viral DNA synthesised (Table 4). Although considerable amounts of viral DNA were present in the cells at 0 time and at 10 h post infection, none of this DNA or an amount below control values, was associated with the cell DNA purified by sedimentation in alkaline sucrose gradients. This further confirms that sedimentation of infected cell DNA in alkaline sucrose gradients is an adequate method for removing free viral DNA from cellular DNA.

DISCUSSION OF RESULTS

The results of the experiments reported in this chapter give evidence that some adenovirus DNA associates with the host DNA during productive infection. Three major problems arise in interpreting these experiments as evidence for integration of viral DNA during productive infection. There is a large amount of intracellular free viral DNA, and there could possibly be as yet undetected fast sedimenting replicative forms of viral DNA. Both of these viral species are potential contaminants of the cell DNA. The third problem is that added free viral DNA is not a completely satisfactory control for noncovalent entanglement of cell and intracellular viral DNA. Such an artefact has not been observed, but cannot be rigorously excluded. To take account of these problems, the cell DNA was separated from the viral DNA in CELO virus infected cells by three methods that rely on independent physical and chemical properties of the DNA.

Sedimentation in alkaline sucrose gradients separates cell from viral DNA on the basis of the difference in their molecular weight. When CELO virus infected cells were lysed with 0.3M NaOH - 0.5 percent SDS and the released DNA was centrifuged on such gradients, the cell DNA sedimented as a main peak at 98S. Contamination of the cell DNA with free viral DNA of mature length due to trapping and sedimentation artefacts, was estimated from a control in which unlabelled CELO virus DNA was added to uninfected cells before lysis and centrifugation. The infected cell DNA tested by DNA-DNA reannealing kinetics contained, after correction for free viral DNA contamination, 500 to 1,000 viral DNA equivalents per diploid amount of cell DNA. The cell DNA isolated from alkaline sucrose gradients was further fractionated by the network technique, which distinguishes the cell DNA from the viral DNA on the basis of highly repeated sequences in the high molecular weight cell DNA. Again, the same number of viral DNA equivalents per diploid amount of cell DNA was found associated with the cell DNA which entered the network. Cell and viral DNA from infected cells were also separated on the basis of their different buoyant densities in neutral and alkaline CsCl gradients after the cell DNA had been prelabelled with BUdR. Again the same type of control was used and showed that contamination of cell DNA by the added free viral DNA was less than 1 percent. The cell DNA was again found to contain about 1,000 viral DNA equivalents per diploid amount of cell DNA when tested by DNA-DNA reannealing kinetics.

Because the control that we used for all three methods was the addition of free viral DNA, we cannot be absolutely sure that the association between cell DNA and viral DNA that we observe is not due to some unexpected, nonspecific trapping peculiar to intracellular viral DNA. However, parental DNA present after adsorption of virus and viral DNA synthesised at 10 h post infection was not detected in the cellular peak

(Table 4). This indicates that non-integrated intracellular viral DNA does not contaminate cellular DNA by nonspecific trapping to any greater extent than added free viral DNA. Moreover, the qualitative and quantitative agreement of the results obtained by three independent methods leads us to conclude that the viral DNA associated with cell DNA from CELO virus infected cells is due to integration into the cell DNA and not to a hypothetical nonspecific entanglement.

With the reservation mentioned above and in the introduction to this chapter, these results confirm the major conclusion of previous workers (Doerfler *et al.*, 1974; Schick *et al.*, 1976; Baczko *et al.*, 1978) that integration of viral DNA into cell DNA occurs in productively infected cells as well as in cells abortively infected or transformed by adenovirus, and suggest that integration of viral DNA is a fairly common event.

There is no evidence however, that integration is required for viral replication. The results from the time course experiment of integration suggest that integration becomes significant (at least within our limits of detection) only when high amounts of newly replicated viral DNA are present and mostly late in infection, when the rate of viral DNA replication is decreasing.

Chapter 3

THE INTEGRATION OF THE DNA OF Ad5 ts125 AND Ad5 ts36 DURING PRODUCTIVE OR ABORTIVE INFECTION

INTRODUCTION

Adenovirus type 5 (Ad5) is a 'non oncogenic' human adenovirus (group C). It does not induce tumours in animals, unless they are immunosuppressed, but transforms non-permissive or semi-permissive cells in culture (Freeman *et al.*, 1976; McAllister *et al.*, 1969). Ad5 replicates productively in human cells, such as KB, HEK, and HeLa cells. The structural properties and the mechanism of replication of Ad5 DNA (molecular weight 23×10^6) have been described previously and are similar to those reported for CELO virus.

Physical and genetic maps of Ad5 DNA have been constructed (Mulder *et al.*, 1974; Grodzicker *et al.*, 1974; Williams *et al.*, 1974; Figure 3). Some temperature sensitive mutants of Ad5 have proved to be very useful in the identification of viral coded functions involved in adenovirus DNA replication and adenovirus induced cell transformation. These mutants are DNA negative, i.e., they cannot replicate their DNA in restrictive conditions (39-40°C), though they produce normal amounts of viral DNA in permissive conditions (32-33°C). They are defective in early function(s) necessary for the initiation of viral DNA replication. The DNA negative ts mutants of Ad5 (Ad5 ts) fall into two complementation groups, represented by Ad5 ts125 (Ensinger and Ginsberg, 1972) and Ad5 ts36 (Williams *et al.*, 1974). Ad5 ts36 and Ad5 ts125 thus define two distinct viral functions in the initiation of viral DNA replication. The ts36 mutation has been mapped within the left 30 percent end of the Ad5 DNA molecule (Sambrook, 1974), while the ts125 mutation has been localised to the right of centre on the Ad5 DNA molecule, between positions 60 and 66 on the DNA physical map (Lewis *et al.*, 1975). Some of the general properties of these two types of mutants have been previously described, but they will be more extensively discussed here.

The Ad5 ts125 gene product is a 72K protein which binds specifically to single stranded DNA (van der Vliet and Levine, 1973) and is thermolabile for continuous binding to single stranded DNA (Levine *et al.*, 1974; van der Vliet *et al.*, 1975). The 72K protein is produced in high concentration during lytic infection (Levine *et al.*, 1974), which suggests that it might have a stochiometric rather than a catalytic function. The 72K protein is also produced in those Ad2 transformed cells which contain that portion of the adenovirus genome which codes for the protein (Levinson *et al.*, 1976). Ad5 ts125 mutant displays a rapid shut-off in viral DNA systhesis upon a shift from the permissive to the non-permissive temperature. This shut-off is most likely caused by a block in the initiation of new rounds of replication since the growth of nascent chains is not inhibited (Ginsberg *et al.*, 1974; van der Vliet and Sussenbach, 1975). On the other hand, antibody to the purified DNA binding protein inhibited chain elongation of viral DNA molecules in isolated nuclei (van der Vliet *et al.*, 1977). A possible explanation of these apparently contradictory results might be that the DNA binding protein is required for both initiation and chain elongation, but that the ts125 mutation inactivates only the first function.

Ad2 DNA replicative intermediates have been isolated as nucleoprotein complexes, and visualised under electron microscope. The single stranded regions of the molecules appeared coated with the 72K DNA binding protein (Kedinger *et al.*, 1978). The 72K protein seems thus directly implicated in adenovirus DNA replication, with a function as yet unknown. Recent investigations on the relative abundancies of early viral m-RNA in cells infected with Ad5 ts125 suggested that the 72K protein might have a regulatory function in the expression of the early viral genes (Carter and Blanton, 1978).

The Ad5 ts36 gene product has not been identified. Replication of viral DNA in Ad5 ts36 cells decreases over a period of 4-6 h after a shift to the restrictive temperature (Levine *et al.*, 1974; van der Vliet and Sussenbach, 1975). The defect again appears to be in initiation of new rounds of DNA replication.

Both Ad5 ts36 and ts125 have altered cell transforming capacity, as compared to wild type Ad5. Ad5 ts36 transforms rat cells with the same frequency as wild type virus at the permissive temperature, but is transformation negative at the non-permissive temperature (Williams *et al.*, 1974). Cells transformed by Ad5 ts36 at the permissive temperature maintain their transformed phenotype when shifted to the restrictive temperature (Williams *et al.*, 1974). This implies that the Ad5 ts36 gene product is necessary for the initiation, but not for the maintenance, of transformation. Ad5 ts125 transforms rat cells with an increased frequency, as compared to wild type Ad5, at both temperatures (Williams *et al.*, 1974; Ginsberg *et al.*, 1974).

Rat cells transformed by group C adenovirus usually contain several copies of viral DNA sequences which represent only a portion of the viral genome (10-90 percent of the whole genome; Gallimore *et al.*, 1974; Sharp *et al.*, 1974; Flint *et al.*, 1976). All transformed cells contain at least the left 7 percent end of the viral genome, which has been shown to be required to establish transformation (Graham *et al.*, 1974). On the other hand, hamster cells transformed by adenoviruses contain 90 to 100 percent of the viral genome (Fanning and Doerfler, 1976; Green *et al.*, 1976; one CELO line has less than this). A number of lines of evidence suggest that the association between viral and cell DNA in transformed cells is covalent, as previously described (Bellett, 1975; Green *et al.*, 1976).

Some lines of rat cells transformed by Ad5 ts125 at permissive, restrictive and intermediate temperature have been examined for their content of viral DNA sequences (Mayer and Ginsberg, 1977). All the HindIII restriction endonuclease fragments of Ad5 DNA were represented in two cell lines of rat cells transformed at the non-permissive temperature (39.5°C) and the Ad5 DNA left-end fragments were slightly ($\leq$2 fold) overrepresented as compared to the DNA right-end fragments. In some lines of cells transformed by Ad5 ts125 at the permissive temperature (32°C) the pattern of the viral DNA sequences was identical to that found in wild type Ad5 transformed cells (at 36°C), and showed that some portion(s) of the viral DNA were absent or markedly under-represented.

Though not proved in the experiments reported, it was assumed that the sequences of viral DNA in the transformed cells were integrated. It was suggested that the increased sequence content of viral DNA, presumably integrated, in Ad5 ts125 transformed cells at 39.5°C was correlated with the increased frequency of transformation shown by Ad5 ts125 (Mayer and Ginsberg, 1977). This correlation does not hold, however, when the patterns of integration of wild type Ad5 DNA at 36°C and of Ad5 ts125 DNA at 32°C are compared. These were almost identical, though the transforming frequency by Ad5 ts125 is also higher than that of wild type virus at the permissive temperature.

It appears therefore that: (1) Ad5 ts36 and Ad5 ts125 mutants are defective in two distinct functions necessary for viral DNA replication, (2) the ts36 gene product is necessary for the establishment of transformation and (3) the ts125 gene product is not necessary to initiate transformation, but indirectly influences the frequency of transformation. Ad5 ts36 and ts125 mutants are therefore convenient for investigating whether any relation exists between integration and replication of viral DNA, and between the frequency or sequence specificity of integration and

the frequency of transformation by adenovirus as suggested by Mayer and Ginsberg (1977). The relationship between integration and adenovirus DNA replication can be investigated by analysis of the viral DNA integrated into cellular DNA during productive infection, in conditions which allow or inhibit the synthesis of the viral DNA (i.e., at the permissive and at the non-permissive temperature). The relationship between frequency and sequence specificity of integration and frequency of transformation can be studied in a similar way, but in an abortive cell system.

Studies on cells abortively infected by DNA tumour viruses are of interest from two points of view. Since some of the survivors become transformed, events during abortive infection may be relevant to these processes. Also, late viral functions are usually not expressed in abortively infected cells, and early viral genes functions can be therefore studied without complication by late viral gene expression.

The best characterised system of abortive infection by adenoviruses is represented by the Ad12-hamster cells system (Strohl, 1969; Doerfler, 1970). In hamster cells Ad12 does not replicate and some of the input viral DNA is integrated. Rat cells have often been used for transformation experiments with Ad2 and Ad5. Rat cells are semipermissive for these viruses: up to 80 percent of the infected cells produce virion antigens, and up to 500 pfu/cell of infectious virus is produced. Most cells die, but transformed cells can arise among the survivors. Mouse cells have never been used for studies on abortive infection and cell transformation with adenoviruses. Experiments designed to characterise the response of mouse cells to infection with Ad5 (and other human and non human adenovirus serotypes) have been done (Younghusband, Tyndall and Bellett, unpublished results). C57Black mouse cells, when infected with Ad5 (5 to 10 i.u./cell) produce not more than 0.1 i.u./cell of

infectious virus two days after infection. No infectious virus can be detected at 9 days after infection. However, viral DNA synthesis occurs in the Ad5 infected mouse cells, as detected by analysis in CsCl equilibrium gradients. Viral DNA synthesis is detected starting from about 30 h post infection and continues for several days. The mouse cells therefore offer a suitable system for investigating abortive infection by adenovirus.

EXPERIMENTS AND RESULTS

THE INTEGRATION OF THE DNA OF Ad5 ts36 AND Ad5 ts125 IN HUMAN EMBRYO KIDNEY CELLS (HEK)

The integration of the DNA of Ad5 mutants ts36 and ts125 was investigated in permissive human embryo kidney (HEK) cells to determine whether viral DNA replication was required for integration. It was of interest also to determine whether the two mutants differed in their capacity to integrate their DNA in permissive cells, at the non-permissive temperature. If either mutant were defective in integration it would implicate a viral gene in this process.

Network Analysis of Viral DNA in HEK Cells infected with Ad5 ts36 and ts125

Because the amount of viral DNA in these experiments was very low, the network method was used. HEK monolayer cultures were inoculated with 3-6 i.u./cell of Ad5 ts125 or Ad5 ts36 and incubated at either 33°C or 38.5°C for 2 h. Medium was then added and the cultures incubated for a further 16 h at the same temperature. DNA was prepared from the cells and analysed by the network method. The total number of copies per cell of viral DNA recovered after 18 h at the non-permissive temperature was

TABLE 5: Network analysis of viral DNA in HEK cells infected with Ad5 ts36 or ts125

HEK cells were infected with mutant virus and incubated for 18 h at the temperature shown. DNA was then extracted and analyzed by the network method. Trapping of free viral DNA in the network was measured (Bellett, 1975) by adding ^{32}P-labelled Ad5 DNA to 5 ml of the sample.

TABLE 5

NETWORK ANALYSIS OF VIRAL DNA IN HEK CELLS INFECTED WITH Ad5 ts 36 AND ts125

MUTANT	TEMPERATURE	FRACTION	CELL DNA (mg)	VIRAL DNA ADDED ^{32}P DNA %	VIRAL DNA TOTAL INTRACELLULAR (ng)	VIRAL DNA STATE	VIRAL DNA COPIES/CELL
ts36	33.0°	NETWORK	6.4	10.1	408	INTEGRATED	6.7
(5.8 pfu/cell)		SUPERNATANT	0 9	89.9	1,429	FREE	36.0
		TOTAL	7.3		1,837	TOTAL	42.7
ts36	38.5°	NETWORK	4.2	6.6	41	INTEGRATED	1.3
(5.8 pfu/cell)		SUPERNATANT	3.2	93.4	163	FREE	3.4
		TOTAL	7.4		204	TOTAL	4.7
ts125	38.5°	NETWORK	5.7	27.5	31	INTEGRATED	0.4
(3 pfu/cell)		SUPERNATANT	1.0	72.5	50	FREE	1.6
		TOTAL	6.7		81	TOTAL	2.0

less than the input multiplicity with both mutants (Table 5), while the corresponding value for ts36 after 18 h at 33°C (equivalent to about 9 h at 38.5°C) was tenfold higher. In all experiments about 15 percent of the viral DNA was in the integrated state as judged by network analysis. However, the number of copies per cell integrated at the non-permissive temperature was lower because the viral DNA had not replicated. Although the number of copies integrated is subject to quantitative errors of at least 10 percent, the results show that neither of the mutants is defective in integration of viral DNA.

THE INTEGRATION OF THE DNA OF Ad5, Ad5 ts36 AND Ad5 ts125 MUTANTS IN MOUSE CELLS

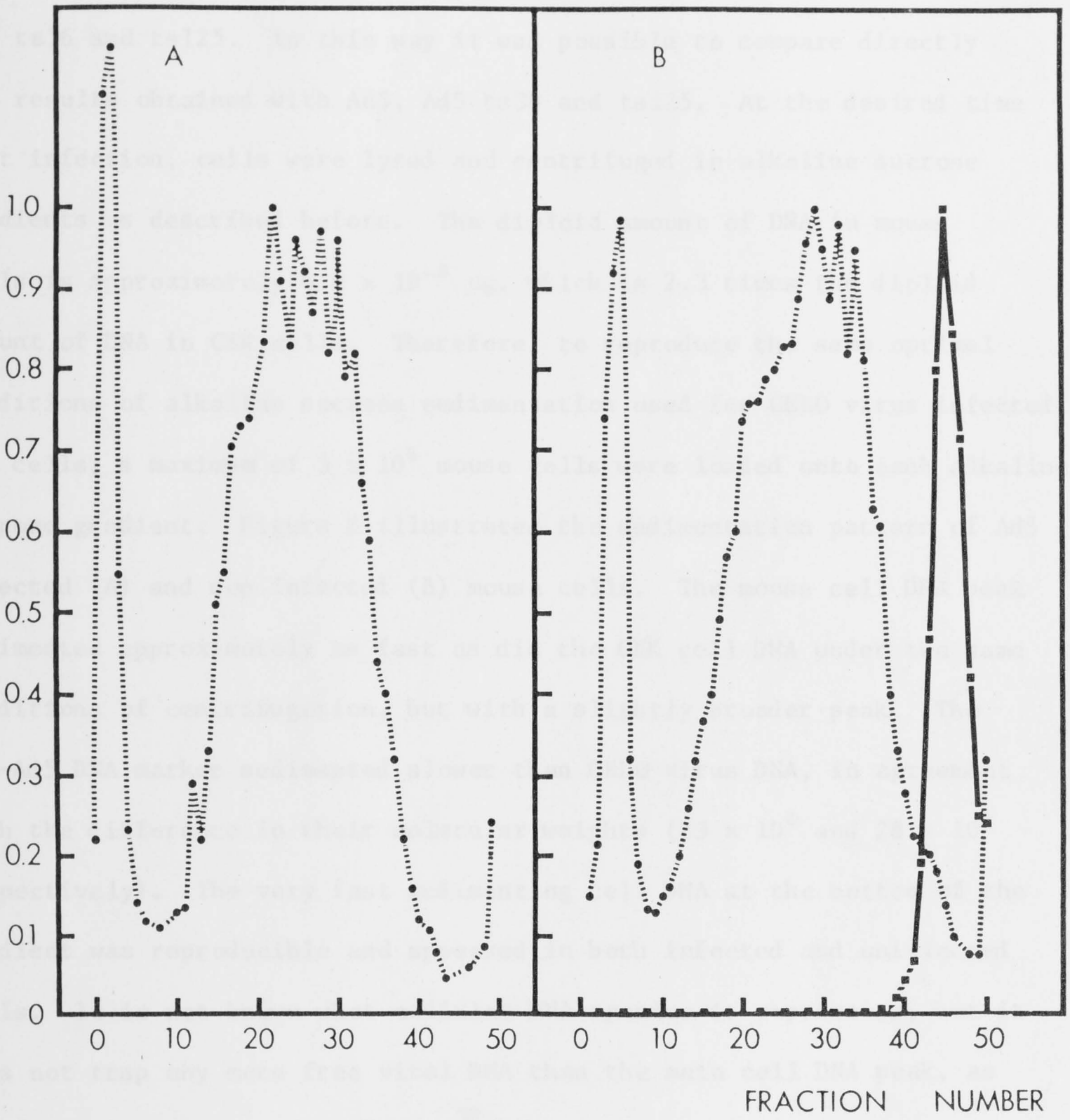
In the previous chapter it was shown that sedimentation in alkaline sucrose gradients is an adequate method of separation of cell DNA plus integrated viral DNA from free viral DNA in adenovirus infected cells. The type of control used for estimating the contamination of the cell DNA by free viral DNA in adenovirus infected cells appeared to be sufficient since replicative viral DNA forms which sediment faster than the strands of virion DNA have not been detected. This method was therefore used to analyse the cell DNA from C57Black mouse cells (J.M.), infected with Ad5 or with its mutants ts36 and ts125. In each case, uninfected mouse cells plus a known amount of free viral DNA were used as a control. The cell DNA from both infected and uninfected cells was tested for viral DNA by reannealing kinetics.

Analysis by Sedimentation in Alkaline Sucrose Gradients of Viral DNA in C57Black Mouse Cells infected with Ad5 Wild Type

^{3}H-dThd-labelled mouse cell monolayers (2/3 confluent) were inoculated with 10 i.u./cell of Ad5 and incubated at 32.5 - 33°C for

FIGURE 8: Alkaline sucrose gradients of mouse cell DNA from (A) infected and (B) uninfected cells

Sedimentation is from right to left. Mouse cells were prelabelled with ^{3}H-dThd prior to infection; (●---●) ^{3}H-cell DNA; (■---■) ^{32}P-Ad5 DNA added as a sedimentation marker.

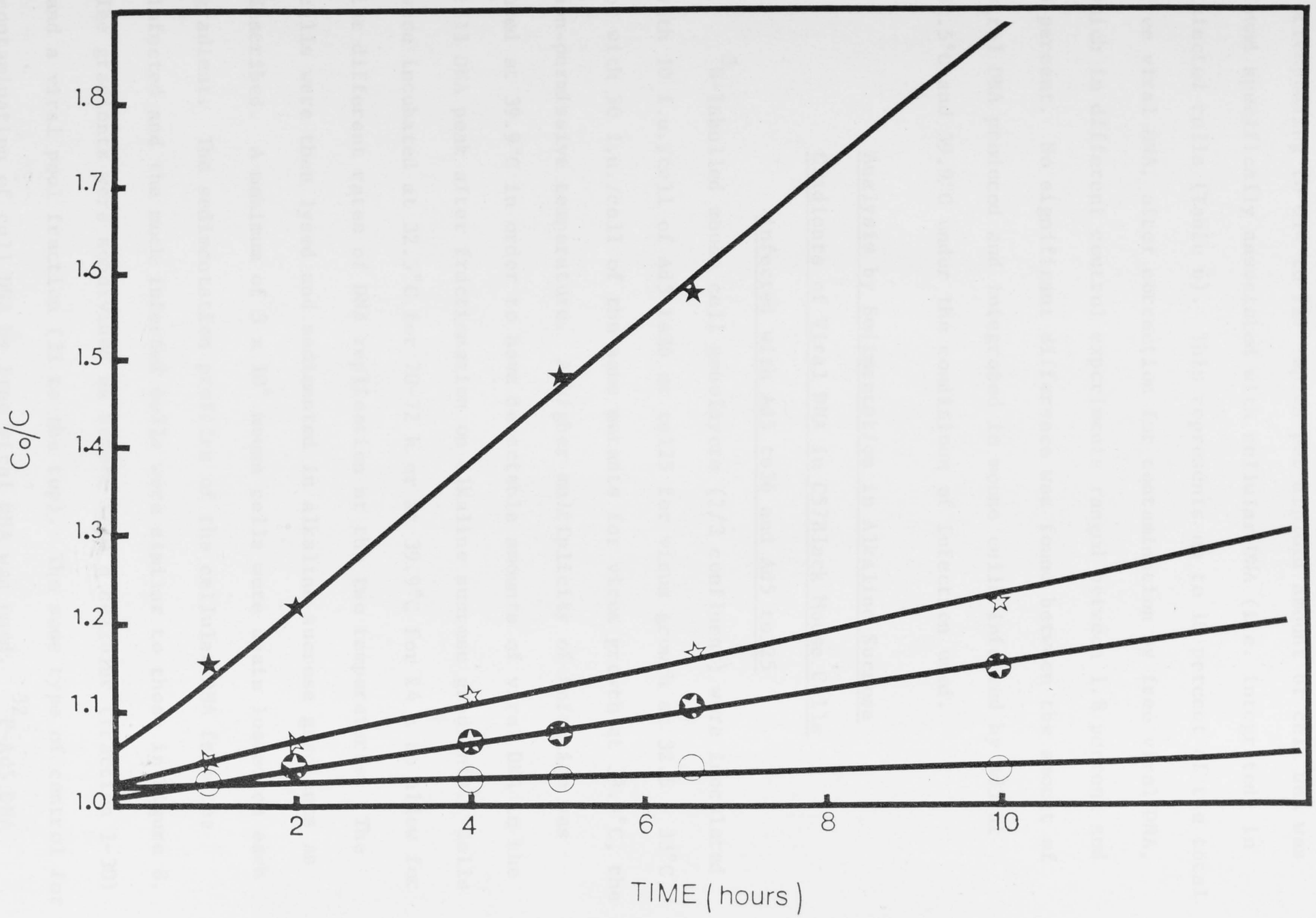

A
B
CPM RELATIVE TO PEAK
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
FRACTION NUMBER

70-72 h. In some experiments cells were inoculated with 50 i.u./cell of Ad5 and incubated at 39.9°C for 24 h. These conditions of infection were the same as those used later when mouse cells were infected with Ad5 ts36 and ts125. In this way it was possible to compare directly the results obtained with Ad5, Ad5 ts36 and ts125. At the desired time post infection, cells were lysed and centrifuged in alkaline sucrose gradients as described before. The diploid amount of DNA in mouse cells is approximately 6.0×10^{-6} µg, which is 2.3 times the diploid amount of DNA in CEK cells. Therefore, to reproduce the same optimal conditions of alkaline sucrose sedimentation used for CELO virus infected CEK cells, a maximum of 3×10^6 mouse cells were loaded onto each alkaline sucrose gradient. Figure 8 illustrates the sedimentation pattern of Ad5 infected (A) and non infected (B) mouse cells. The mouse cell DNA peak sedimented approximately as fast as did the CEK cell DNA under the same conditions of centrifugation, but with a slightly broader peak. The ^{32}P-Ad5 DNA marker sedimented slower than CELO virus DNA, in agreement with the difference in their molecular weights (23×10^6 and 28×10^6 respectively). The very fast sedimenting cell DNA at the bottom of the gradient was reproducible and appeared in both infected and uninfected cells. It is not known what cellular DNA species it represents, but it does not trap any more free viral DNA than the main cell DNA peak, as detected by sedimentation of the ^{32}P-viral DNA marker and by reannealing kinetics of Ad5 DNA.

The gradient was usually divided into two pools: the cellular (fraction 1-30) and the viral (fractions 31 to the top). Each of them was processed as described previously and then tested for viral DNA by ^{32}P-labelled Ad5 DNA reannealing kinetics (Figure 9). In parallel experiments mock-infected mouse cells, to which a known amount of Ad5 DNA

FIGURE 9: Reannealing kinetics of ^{32}P-Ad5 DNA (1.6 ng) in the presence of Ad5 infected mouse cell DNA isolated from alkaline sucrose gradients

Mouse cells were infected with Ad5 at a multiplicity of 10 i.u./cell. After 72 h at 32.5°C the cells were lysed as described and centrifuged on alkaline sucrose gradients. Fractions 1 to 30 and 31 to the top (Figure 8) were pooled and tested for viral sequences by reannealing kinetics. Reannealings were done as described in legend to Figure 6. The reannealings followed second order kinetics and linear regression lines were calculated by the least squares method. Reannealing kinetics of ^{32}P-Ad5 DNA in the presence of calf thymus DNA only ◯, calf thymus DNA plus 20 ng of unlabelled Ad5 DNA ☆, calf thymus DNA plus DNA from pool 1-30 ✪, calf thymus DNA plus DNA from pool 31 to the top ★.


1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
Co/C
2
4
6
8
10
TIME (hours)

had been added, were processed and analysed in the same way. Viral DNA corresponding to 614 to 727 copies per diploid amount of cell DNA was found specifically associated with cellular DNA (i.e. integrated) in infected cells (Table 6). This represents up to 10 percent of the total free viral DNA, after correction for contamination by free viral DNA, which in different control experiments ranged between 1.8 percent and 3 percent. No significant difference was found between the amount of viral DNA produced and integrated in mouse cells infected by Ad5 at 32.5°C and 39.9°C under the conditions of infection used.

Analysis by Sedimentation in Alkaline Sucrose Gradients of Viral DNA in C57Black Mouse Cells infected with Ad5 ts36 and Ad5 ts125

^{3}H-labelled mouse cell monolayers (2/3 confluent) were inoculated with 10 i.u./cell of Ad5 ts36 or ts125 for virus growth at 32.5 - 33°C, or with 50 i.u./cell of the same mutants for virus growth at 39.9°C, the non-permissive temperature. A higher multiplicity of infection was used at 39.9°C in order to have detectable amounts of viral DNA in the cell DNA peak after fractionation on alkaline sucrose gradients. Cells were incubated at 32.5°C for 70-72 h or at 39.9°C for 24 h to allow for the different rates of DNA replication at the two temperatures. The cells were then lysed and sedimented in alkaline sucrose gradients as described. A maximum of 3×10^{6} mouse cells were again loaded on each gradient. The sedimentation profiles of the cellular DNA from the infected and the mock infected cells were similar to those in Figure 8. The gradients were subdivided as before into a cellular (fraction 1-30) and a viral pool fraction (31 to the top). The same type of control for contamination of cell DNA by free viral DNA was used. ^{32}P-Ad5 DNA reannealing kinetics were used to estimate the amounts of viral DNA associated with the viral and the cell DNA.

TABLE 6: Quantitative analysis of adenovirus DNA associated with infected mouse cell DNA after alkaline sucrose gradient sedimentation

Mouse cells prelabelled with ^{3}H-dThd were infected with wild type or mutant virus under the indicated conditions. Cells were then lysed, centrifuged on alkaline sucrose gradients, and processed as described in Fig. 8. Cellular (fractions 1-30) and viral (fractions 31 to the top) peaks were tested for viral DNA by reannealing kinetics: the amounts of viral DNA calculated from reannealing kinetics were corrected for the recovery of ^{3}H counts as described in legend to Table 1. The amount of viral DNA in the cell peak was corrected for contamination by free viral DNA as determined from control gradients.

In control gradients a known amount of unlabelled Ad5 DNA was added to uninfected mouse cells, which were then lysed, centrifuged in alkaline sucrose gradients and processed as the infected cells. The fractions corresponding to viral and cellular DNA peaks were collected as with the infected cells, and then tested for viral sequences by reannealing kinetics. Free viral DNA contaminating the cell DNA accounted from 1.8 to 3 percent of the total viral DNA.

The number of copies per cell represents the number of viral DNA equivalents per diploid amount of cell DNA, assuming that Ad5 DNA has a molecular weight of 23×10^{6} and the diploid amount of DNA in mouse cells is 6×10^{-6} µg. In the table: viral DNA in cell peak (copies/cell) = integrated viral DNA = infected cell value-control.

The amounts of viral DNA shown have been normalized to those obtained after infection of 2×10^{6} mouse cells. For more accurate estimates of the viral DNA present in both viral and cellular peaks during infection by mutant virus at 39.9°C about 12×10^{6} cells were actually infected.

For method of calculation of viral DNA in copies per cell and example see p126a

TABLE 6

QUANTITATIVE ANALYSIS OF ADENOVIRUS DNA ASSOCIATED WITH MOUSE CELL DNA AFTER ALKALINE SUCROSE GRADIENT SEDIMENTATION

VIRUS	CONDITIONS	VIRAL DNA IN VIRAL PEAK (μG)	VIRAL DNA IN CELL PEAK		% OF VIRAL DNA IN CELL PEAK
			(μG)	COPIES/ CELL	
AD5 WT	10 IU/CELL, 72H P.I. 32.5°C	1.75	0.140	727	8
AD5 WT	50 IU/CELL, 24H P.I. 39.9°C	1.80	0.180	614	10
AD5 TS36	10 IU/CELL, 70H P.I. 32.5°C	2.30	0.160	588	7
AD5 TS36	50 IU/CELL, 24H P.I. 39.9°C	0.030	0.004	15	13
AD5 TS125	10 IU/CELL, 72H P.I. 32.5°C	0.050	0.004	40	8
AD5 TS125	50 IU/CELL, 24H P.I. 39.9°C	0.013	0.0013	9	10

Surprisingly, Ad5 ts125 appeared to be defective in DNA replication in mouse cells at 32.5°C while Ad5 ts36 DNA was produced in approximately the same amount as Ad5 wild type DNA (Table 6). Ad5 ts125 grew to very high titers in human HEK cells at 32.5°C, and Ad5 ts125 DNA was efficiently replicated (about 4 μg/2 x 10^6 cells at 24 h post infection) in human KB cells at the same temperature. The amount of viral DNA produced in Ad5 ts36 infected mouse cells was almost 80 fold higher at 32.5°C than at 39.9°C. There was only a 3-4 fold increase in the amount of viral DNA present at 32.5°C in Ad5 ts125 infected mouse cells (Table 6). However, both mutants integrated viral DNA to approximately the same extent at both temperatures. The percentage of viral DNA integrated into mouse cell DNA ranged from 7 percent at 32.5°C to 13 percent at 39.9°C for ts36, and from 8 percent at 32.5°C to 10 percent at 39.9°C for ts125. Also the percentage of viral DNA integrated in Ad5 wild type infected mouse cells was between 8 percent and 10 percent. However, the number of copies per cell integrated was lower for both mutants at the non-permissive temperature and for ts125 at 32.5°C since the viral DNA was not replicated. These results indicate that neither mutant is defective in integration at either temperature during abortive infection of mouse cells.

Analysis of Restriction Endonuclease Fragments of Ad5 DNA integrated in Mouse Cells infected with Ad5 Wild Type and Ad5 ts36

The above experiments show that the percentage of viral DNA integrated in mouse cells infected by the Ad5 ts mutants at the non-permissive temperature is not very different from that found at the permissive temperature or that found for whild type Ad5 at either temperature. It appears that the failure of Ad5 ts36 to transform cells at the non-permissive temperature is not due to a failure to integrate. However

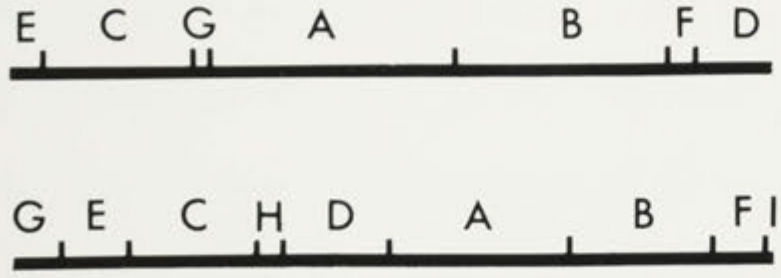
it cannot be ruled out that at the permissive and at the non-permissive temperature the viral DNA integrates with different patterns in mouse cells infected with Ad5 ts36. The left hand 7 percent of the Ad5 DNA, which contains the sequences necessary for cell transformation by Ad5 (Graham *et al.*, 1974) might be underrepresented, or absent among the viral DNA sequences integrated at the non-permissive temperature, without this being detected by reannealing kinetics with whole ^{32}P-Ad5 DNA. The failure of the left hand end of the Ad5 DNA to integrate might be responsible for the inability of Ad5 ts36 to transform at 39.9°C. It was therefore important to determine whether all parts of the Ad5 DNA genome were equally represented among the viral sequences integrated in mouse cells infected with Ad5 ts36, at both permissive and non-permissive temperature. The sequences of Ad5 DNA integrated in mouse cells infected with wild type Ad5 at 32.5°C and at 39.9°C were also analysed in the same way as a control for the results with ts36, and to test the suggestion of Mayer and Ginsberg (1977) that the Ad5 sequences integrated are restricted in cells infected by wild type virus, but not in cells infected by ts125.

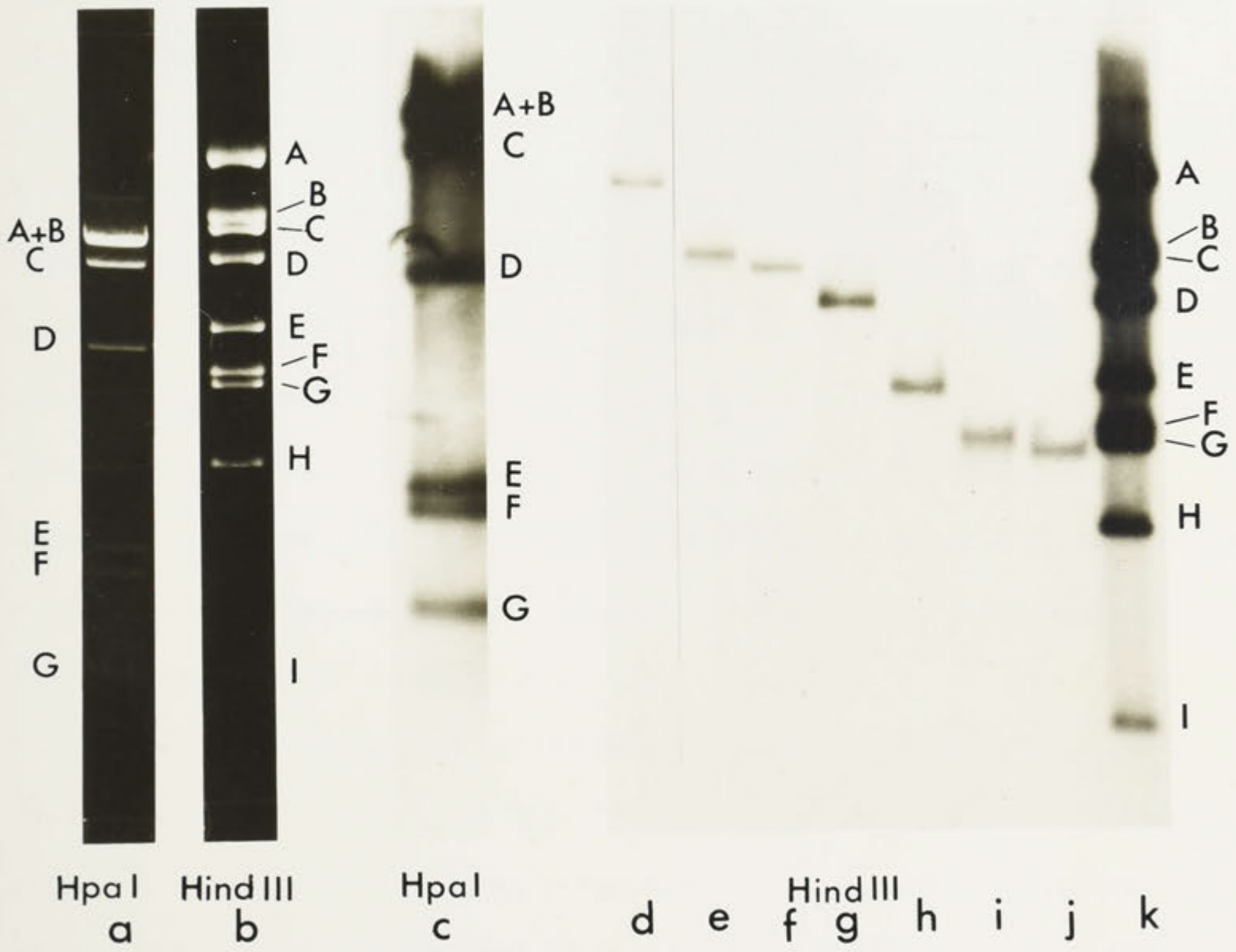
(i) Analysis of HpaI restriction fragments of Ad5 DNA integrated in mouse cells infected with Ad5 wild type and Ad5 ts36.

^{32}P-labelled Ad5 DNA of high specific activity was cleaved with restriction endonuclease, HpaI. The DNA fragments were separated by electrophoresis on agarose gels (Figure 10) and purified as described in the Materials and Methods section. Each ^{32}P-Ad5 DNA HpaI fragment was reannealed in the presence of cell DNA purified by alkaline sucrose gradient sedimentation from mouse cells infected by Ad5 ts36 at 32.5°C. The number of copies of each of the viral DNA fragments was calculated as described by Gelb *et al.* (1971). A similar analysis using DNA from

FIGURE 10: Gel electrophoresis of Ad5 DNA cleaved by restriction endonuclease HpaI or HindIII

The upper part of the figure shows the HpaI (Mulder *et al.*, 1974) and HindIII (Sambrook and Sussenbach, as reported by Mayer and Ginsberg, 1977) restriction maps of Ad5 DNA. In the lower part of the figure, Ad5 DNA (1-2 µg) was cut with HpaI (a) or HindIII (b), and the resulting fragments were fractionated by electrophoresis on 0.8 percent agarose gels. The DNA fragments were visualised under ultraviolet (U.V.) illumination after staining with ethidium bromide (a and b). For preparative separation, ^{32}P-Ad5 DNA (2-6 µg) was cleaved with HpaI or HindIII, and the bands were located and cut under U.V. illumination, after staining with ethidium bromide. The DNA bands could also be identified by autoradiography, after cleavage of DNA with HpaI (c) or HindIII (k). The separated fragments were eluted from the gel as described in Materials and Methods. Purity of the separated fragments was checked by gel electrophoresis and autoradiography. Lanes d to j show the purity of some of the HindIII fragments of ^{32}P-Ad5 DNA, as obtained after they have been cut and eluted from the gel. The fragments shown are: A(d), B(e), C(f), D(g), E(h), F(i), and G(j). When necessary, each DNA fragment was further purified by re-electrophoresis on agarose gels.


E C G A B F D
HpaI
G E C H D A B F I
HindIII


A+B
C
D
E
F
G
H
I
HpaI
HindIII
HpaI
HindIII
a
b
c
d
e
f
g
h
i
j
k

cells infected by ts36 at 39.9°C was not possible because insufficient viral DNA was present. However, this was done in a later experiment using endonuclease HindIII (Table 8).

All the HpaI fragments of Ad5 DNA were represented in approximately equimolar amounts in cell DNA from mouse cells infected by Ad5 ts36 at 32°C. The average number of copies of each fragment per cell was 670. This value was not significantly different from the number of viral DNA equivalents (588; Table 6) detected in the same cell DNA by reannealing kinetics with the whole Ad5 DNA. The amount of each Ad5 DNA HpaI fragment present in the viral DNA peak, as isolated from centrifugation in alkaline sucrose gradients of Ad5 ts36 infected cells at 32.5°C, was also estimated by reannealing kinetics. Since fragments of viral DNA should be represented in equimolar amounts in virion size DNA, reannealing kinetics with the free viral DNA produced during Ad5 ts36 infection of mouse cells at 32.5°C, serves as an internal control. As expected, all fragments of viral DNA were present in approximately equal amounts, corresponding, on the average, to 8.14×10^{10} copies of each fragment per 2×10^{6} cells (Table 7A).

Combinations of equimolar amounts of ^{32}P-Ad5 DNA HpaI fragments, representing the left 24 percent (L), the middle 60 percent (M), and the right 14 percent (R) portions, respectively of the Ad5 DNA genome, were used as probes for reannealing kinetics in the presence of cell DNA purified by alkaline sucrose sedimentation from mouse cells infected by Ad5 wild type at either 32.5°C or 39.9°C (Table 7B). All parts of the viral genome were present in the cell DNA from mouse cells infected by Ad5 at either temperature. The sequences mapping within the left 24 percent end of Ad5 DNA appeared slightly overrepresented in the DNA of cells infected at 39.9°C, while the sequences mapping within the middle 60 percent portion of Ad5 DNA appeared slightly underrepresented in the DNA of

TABLE 7: Quantitative analysis of HpaI restriction endonuclease fragments of Ad5 DNA associated with infected mouse cell DNA following alkaline sucrose gradient sedimentation

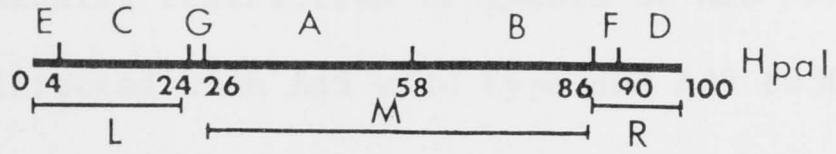
Mouse cells, prelabelled with ^{3}H-dThd, were infected by wild type or mutant virus under the indicated conditions. Cells were then lysed, centrifuged on alkaline sucrose and processed as described. Each ^{32}P-labelled HpaI restriction fragment, or a combination (L, M, and R) of equimolar amounts of fragments, was reannealed in the presence of the cellular (fractions 1-30) and viral (fractions 31 to the top) DNA peaks from infected cells. The results in section B of the table have been obtained from reannealing kinetics in the presence of the cell DNA peak only. The amounts of viral DNA sequences calculated from reannealing kinetics were corrected for recovery of ^{3}H counts as described for Table 1.

A control of uninfected cell DNA plus added viral DNA was used as usual to estimate contamination of the cell DNA peak by free viral DNA. It was assumed that contaminating free viral DNA contained equimolar amounts of each fragment to calculate the appropriate correction factors for the fragments. The number of Ad5 DNA equivalents (right) were calculated by reannealing kinetics with whole ^{32}P-Ad5 DNA. The table includes (top) a map of the HpaI restriction fragments of Ad5 DNA (Mulder *et al.*, 1974). The numbers on the map are percentages of the distance from the left-hand end (0).

For method of calculation of viral DNA in copies per cell and example see p126a

TABLE 7

QUANTITATIVE ANALYSIS OF HpaI RESTRICTION ENDONUCLEASE FRAGMENTS OF Ad5 DNA ASSOCIATED WITH INFECTED MOUSE CELL DNA FOLLOWING ALKALINE SUCROSE GRADIENT SEDIMENTATION



A

VIRUS	CONDITIONS	NO. OF EQUIVALENTS OF EACH HPAI RESTRICTION FRAGMENT OF AD5 DNA PER CELL					NO. OF EQUIVALENTS OF AD5 DNA PER CELL
		E	C	A+B	F	D	
AD5 ts36 (CELL PEAK)	32.5°C, 70H P.I. 10 IU/CELL	576	792	604	650	743	588
AD5 ts36 (VIRAL PEAK)[a]	" " "	950	850	870	550	900	600[a]

B

VIRUS	CONDITIONS	NO. OF EQUIVALENTS OF EACH GROUP OF HPAI RESTRICTION FRAGMENTS OF AD5 DNA PER CELL		
		L	M	R
AD5 WT	32.5°C, 70H P.I. 10 IU/CELL	900	650	1000
AD5 WT	39.9°C, 24H P.I. 50 IU/CELL	1300	850	1050

a Total number of equivalents ($\times 10^{-8}$) of viral DNA fragments

cells infected at 32.5°C (Table 7B). However, a difference less than twofold in the molar amounts of these sequences is unlikely to be significant and was not found in later experiments using endonuclease HindIII. Sequences from every part of Ad5 DNA therefore appear to be integrated in mouse cells abortively infected by either Ad5 or Ad ts36. The pattern of integration is approximately the same in both cases.

(ii) Analysis of HindIII restriction fragments of Ad5 DNA integrated in mouse cells infected with Ad5 wild type and Ad5 ts36.

^{32}P-labelled Ad5 DNA of high specific activity was digested with HindIII restriction endonuclease, and the DNA fragments were fractionated by electrophoresis on agarose gels (Figure 10).

At 39.9°C, the amount of viral DNA in cells infected with Ad5 ts36 is very low, and sufficiently large amounts of infected cell DNA could not be obtained by alkaline sucrose gradient sedimentation to allow analysis of integrated sequences using each HindIII restriction fragment. Therefore, combinations of equimolar amounts of HindIII restriction fragments of Ad5 DNA representing the left end 32 percent (L), the middle 38 percent(M), and the right end 27 percent (R) of the Ad5 DNA molecule, were used as probes in reannealing kinetics with the above cell DNA (Table 8). The number of copies of each set of fragments was calculated as described by Gelb *et al.* (1971). At 39.9°C the left, middle and right portions of the viral genome were integrated in mouse cells infected with Ad5 ts36, in approximately equimolar amounts. The same pattern of integration was also observed in Ad5 wild type infected mouse cells (Tables 7B and 8).

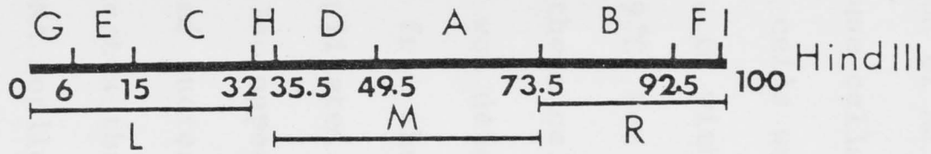
These results show that the full complement of the Ad5 DNA sequences is integrated during the abortive infection of mouse cells with Ad5 wild type and that the same pattern of viral DNA integration is observed

TABLE 8: Quantitative analysis of HindIII restriction endonuclease fragments of Ad5 DNA associated with infected mouse cell DNA following alkaline sucrose gradient sedimentation

Mouse cells, prelabelled with ^{3}H-dThd, were infected by wild type or mutant virus under the indicated conditions. Cells were then lysed, centrifuged on alkaline sucrose gradients, and processed as described. Combinations (L, M, and R) of equimolar amounts of HindIII fragments of ^{32}P-Ad5 DNA were reannealed in the presence of DNA from the cell peak (fraction 1-30, Figure 8). The amounts of viral sequences calculated from reannealing kinetics were corrected for recovery of ^{3}H counts as described in legend to Table 1. The same control as that described in legend to Table 7 was used to estimate contamination of the cell DNA peak by free viral DNA. The number of equivalents of Ad5 DNA per cell (right) were calculated by reannealing kinetics using whole ^{32}P-Ad5 DNA. The table includes a map of the HindIII restriction fragments of Ad5 DNA (Sambrook and Sussenbach, reported by Mayer and Ginsberg, 1977). The numbers on the map are percentages of the distance from the left-hand end (0).

TABLE 8

QUANTITATIVE ANALYSIS OF HindIII RESTRICTION ENDONUCLEASE FRAGMENTS OF AD5 DNA ASSOCIATED WITH INFECTED MOUSE CELL DNA FOLLOWING ALKALINE SUCROSE GRADIENT SEDIMENTATION



VIRUS	CONDITIONS		NO. OF EQUIVALENTS OF EACH GROUP OF HindIII RESTRICTION FRAGMENTS OF AD5 DNA PER CELL			NO. OF EQUIVALENTS OF AD5 DNA PER CELL
			L	M	R	
AD5 ts36	39.9°C 24 h p.i. 50 iu/cell	Exp. 1	6.3	9.7	11.4	9.8
		Exp. 2	7.3	12.3	9.2	15
AD5 WT	32.5°C 70 h p.i. 10 iu/cell		591	640	620	700

when the cells are infected with Ad5 ts36 at both 32.5°C and 39.9°C. They also suggest that the failure of Ad5 ts36 to transform at 39.9°C is not due to a failure to integrate the left end (transforming) portion of the viral DNA.

DISCUSSION OF RESULTS

The integration of Ad5 and of its mutants ts36 and ts125 was investigated in mouse cells, which are abortively infected by group C adenoviruses. The cells were infected at the appropriate temperature with either mutant, or with Ad5 wild type. Ad5 replicated its DNA in mouse cells at 39.9°C, but neither mutant did so. Both Ad5 and Ad5 ts36 DNA replicated in these cells when infected at the permissive temperature. However, Ad5 ts125 was defective in DNA replication in mouse cells at 32.5°C. Ad5 ts125 from the same stock used to infect mouse cells, grew efficiently and replicated its DNA in human cells at the permissive temperature. It also appeared that Ad5 ts125 stocks which contained revertants, produced increased amounts of viral DNA in mouse cells at 32.5°C. This indicates that the ts125 mutation is responsible for the restricted viral DNA replication in mouse cells. No explanation for this phenomenon can be given at present. The 72K protein, produced in Ad5 ts125 infected permissive cells at the permissive temperature appears to be more thermolabile for binding to single stranded DNA than the wild type protein (Levine *et al.*, 1974). The possibility that the mutated protein can be complemented at the permissive temperature by some thermosensitive cellular factor(s) present in permissive human cells and not in non-permissive mouse cells cannot be excluded. However, it is more likely that the ts125 mutation itself has more effect in non permissive than permissive cells at 32.5°C, i.e., the mutation causes a

host range defect in DNA replication as well as a ts defect. In this respect, it is interesting to note that Ad5 ts125 transforms rat cells more frequently than w.t. virus at the permissive as well as the non-permissive temperature, although viral DNA replication in human cells is defective only at the non-permissive temperature.

To investigate the integration of the viral DNA in mouse cells infected with Ad5 or either mutant virus, the infected cell DNA was purified by alkaline sucrose gradient sedimentation. The efficiency of this method for separating cell DNA from free adenovirus viral DNA, when this is present in large amounts, has been shown previously. Wild type and both mutant viruses integrated the same proportion of their DNA at permissive and non-permissive temperatures. From 7 to 13.1 percent of the viral DNA present was integrated into the DNA of mouse cells under all conditions, although in cells infected by either mutant at 39.9°C or ts125 at 32.5°C the absolute number of copies of viral DNA integrated per cell was less than that in cells infected by wild type virus because the total amount of viral DNA was less. The results suggest that the difference in transformation frequency at 39.9°C between Ad5 ts36 and ts125 is not due to a similar difference in integration frequency.

In mouse cells infected with Ad5 ts35 at 39.9°C, the pattern of integration of the viral DNA sequences is similar to that of ts36 DNA at 32.5°C or wild type Ad5 DNA in the same cells. The left, middle and right portions of the genome appear to be equally represented within experimental error among the integrated sequences. This suggests that the failure of Ad5 ts36 to transform non-permissive cells at 39.9°C is not due to a failure to integrate the viral DNA or the transforming portion of the viral DNA. The transforming capacity of Ad5 ts36 was tested in rat cells (Ginsberg *et al.*, 1974). It is possible however, to

extrapolate to rat cells the results obtained with mouse cells since Ad5 ts36 abortive infection of mouse and rat cells is similar.

Investigations on the sequence specificity of integration of Ad5 ts125 in mouse cells, at both 32.5°C and 39.9°C, have been hindered by the almost complete lack of replication of the viral DNA in these cells. In several attempts insufficient cell DNA-associated viral DNA was recovered from ts125 infected cells to allow analysis of the integrated sequences using restriction endonuclease fragments of ^{32}P-viral DNA. However, although the 72K protein is in some respects non functional in mouse cells infected with Ad5 ts125 at either temperature, the same proportion of the input to viral DNA or of the very small amount of newly replicated viral DNA was integrated as in mouse cells infected with Ad5 ts36 or Ad5 wild type. The obvious interpretation of this result is that the ts125 protein is not directly involved in integration.

This conclusion is in disagreement with a previous suggestion by Mayer and Ginsberg (1977) about the role of the ts125 protein in integration and transformation. They proposed that the functional wild type 72K protein reduces the frequency of transformation by decreasing the frequency of integration and also by restricting the viral DNA sequences that can be stably integrated mainly to part of the genome from the left-end. They suggested that the ts125 mutation removes these restrictions, allowing more frequent and more extensive integration. However, since the left 7 percent end of the Ad5 genome contains the only sequences required for transformation, it seems unlikely that the presence of additional sequences from other parts of the genome would increase the frequency of transformation. Moreover, several predictions of the hypothesis proposed by Mayer and Ginsberg are incompatible with my results. If their suggestion were correct, then integration experiments

with DNA from cells infected by wild type Ad5 using whole ^{32}P-viral DNA as probe should show an apparently low integration rate compared with ts125, and restriction of the sequences integrated would result in deviations from second order kinetics in the annealing curves (Frankel *et al.*, 1972). Similarly, restriction of the viral DNA sequences integrated in cells infected by wild type Ad5 should be detected using restriction fragments of ^{32}P-viral DNA as probes. None of these effects was observed (Figure 9). It therefore seems likely that the influence of the ts125 mutation on transformation frequency is due to some other more indirect effect.

The integration of Ad5 ts36 and ts125 has also been investigated in human HEK cells which are permissive for Ad5. The network method was used this time to separate the cell DNA from free viral DNA as an alternative to centrifugation in alkaline sucrose gradients. To avoid contamination of the network fraction by free viral DNA at high concentration, the analysis was done early (18 h after adding virus) at the permissive temperature. The results obtained suggest, in agreement with those reported during infection of mouse cells, that the percentage of viral DNA integrated in cells infected by the mutants at the non-permissive temperature is not very different from that found at the permissive temperature or that found for wild type virus in mouse cells, and that viral DNA replication is not required for integration. Previous studies on abortive infection of hamster cells with Ad12 also showed that, although the viral DNA did not replicate in those cells, it was nevertheless integrated (Doerfler, 1970).

Chapter 4

DISCUSSION OF RESULTS

The results of investigations into the integration of adenovirus DNA during productive and abortive infection have been reported in this thesis. CELO virus, an oncogenic avian adenovirus, and Ad5, a non-oncogenic human serotype, have been chosen as convenient models for studying the integration of adenovirus DNA during productive infection. In order to prove the integration of viral into cellular DNA, it is necessary to show that viral sequences are covalently associated with cellular DNA sequences. During productive infection, large amounts of viral DNA are produced. It is possible to separate cellular DNA from free viral DNA by exploiting the differences in their physical properties, such as molecular weight, buoyant density and sequences reiteration. The cellular DNA can be separated from viral DNA by velocity sedimentation in sucrose gradients, buoyant density sedimentation or network formation (Varmus *et al.*, 1973).

Alkaline conditions during sedimentations will disrupt non covalent bonds. However, some contamination of the cellular DNA by free replicating or mature viral DNA can occur during the separation procedure, and must be taken into account when the cell DNA is tested for integrated viral DNA sequences. Contamination of cellular DNA by as yet undetected forms of replicating viral DNA is extremely unlikely; viral DNA sedimenting faster than virion size DNA in alkaline sucrose gradient, for instance covalently continuous concatemeric or circular viral DNA forms, have never been detected by pulse labelling during adenovirus DNA replication (Horwitz, 1971; Pearson and Hanawalt, 1971; Bellett and Younghusband, 1972; Sussenbach and van der Vliet, 1972; Pettersson, 1973; Pearson, 1975). However, hypothetical concatemeric and fast sedimenting viral DNA forms, which could cosediment with cellular DNA during velocity sedimentation in sucrose gradients, would be separated from cellular DNA

during buoyant density gradient sedimentation. For this reason, I decided to purify cellular DNA from CEK cells productively infected by CELO virus both by velocity and by buoyant density sedimentation. In addition, cellular DNA prepared from infected cells by alkaline sucrose gradient sedimentation, was further fractionated by the network technique, which is thought to test for covalent integration of viral DNA (Varmus *et al.*, 1973; Bellett, 1975). The network technique distinguishes the cell DNA from the viral DNA on the basis of highly repeated sequences in the high molecular weight cell DNA.

Cell DNA from CELO virus infected CEK cells purified by one of these methods, or by a combination of them, was tested for viral DNA sequences by DNA-DNA reannealing kinetics (Gelb *et al.*, 1971). Comparable amounts of viral DNA, corresponding to 500-1,000 equivalents of viral DNA per cell, were specifically associated with the infected cell DNA. These values have been obtained after correction for contamination of cellular DNA by free viral DNA. This contamination was accounted for by control experiments where labelled and unlabelled viral DNA were added to uninfected CEK cell.

The qualitative and quantitative agreement of the results obtained by three independent methods clearly indicates that the viral DNA associated with the cell DNA from CELO virus infected cells is due to integration into the cell DNA, and not to a hypothetical nonspecific trapping, or to as yet undetected forms of replicating viral DNA. Also, each of the methods of purification employed appears to be adequate, with the type of control used, for a quantitative analysis of the state of adenovirus DNA within the infected cells. It is therefore concluded that adenovirus DNA integrates into cellular DNA during productive infection. Experiments on the time course of integration show that integration is not detectable at 10 h post infection, but is detectable

at 18 h post infection, and that its rate increases at late times in infection.

In order to understand whether viral DNA replication is necessary for integration of viral DNA in lytically infected cells, the integration of Ad5 ts36 and ts125 DNA was investigated in human HEK cells. These mutants cannot replicate their DNA at the non-permissive temperature. The network method was used to fractionate HEK cell DNA infected by either mutant at the permissive or the non-permissive temperature. Approximately the same percentage of viral DNA became integrated in the cell DNA in cells infected by either mutant at the non-permissive temperature and was not very different from that found at the permissive temperature or that found in CEK cells infected by wild type CELO virus. This suggests that viral DNA replication is not necessary for integration.

The integration of Ad5 and of its mutants ts36 and ts125 was also investigated in abortively infected mouse cells. As previously described, the two mutants are DNA negative at the non-permissive temperature (Williams *et al.*, 1974; Ginsberg *et al.*, 1974). Ad5 ts36 is also transformation negative at this temperature (Williams *et al.*, 1974), while Ad5 ts125 transforms with increased frequency as compared to Ad5 wild type, at both permissive and non-permissive temperature (Ginsberg *et al.*, 1974). It was of interest to test whether Ad5 ts36 was also negative in integration at the non-permissive temperature and how it compared, with respect to its integrative properties, with Ad5 ts125 and Ad5 wild type. Abortively infected cells were chosen for these investigations since a small proportion of them can become transformed, and, if altered ability to integrate is responsible for altered transformation capacity of the mutant viruses, this is most likely to be detected in abortively infected cells.

The previous experiment on integration during lytic infection showed that sedimentation in alkaline sucrose gradients, plus the control used for contamination by free viral DNA, was an adequate method of separation of cell from viral DNA. This method removed intracellular free viral DNA from the cell DNA, as seen in cell DNA prepared from CELO infected CEK cells at 0 h and 10 h post infection (Table 4). It was therefore suitable for investigating the integration of Ad5 ts mutants DNA into cellular DNA at the non-permissive temperature, at which very small amounts of free viral DNA are present.

DNA from mouse cells infected with either mutant or Ad5 wild type virus at both permissive and non-permissive temperature, was sedimented in alkaline sucrose gradients and the cell peak was tested for viral sequences by reannealing kinetics. The usual type of control was used to allow for contamination of cell by free viral DNA. No significant difference was found between the percentage of viral DNA integrated by each mutant at either temperature, or between the percentage of viral DNA integrated by each mutant and that by wild type Ad5. It appeared also that different parts of the Ad5 ts35 genome were nearly equally represented among the sequences integrated at the non-permissive temperature.

The results on integration of adenovirus DNA during lytic infection presented here are in agreement with other reports on the same topic (Doerfler *et al.*, 1974; Burger and Doerfler, 1974; Schick *et al.*, 1976). However, the experiments by these workers did not rigorously take into account the problem of contamination of the cellular DNA which was tested for viral DNA sequences, by free viral DNA present in large amounts in productively infected cells. The viral DNA was also detected mainly in an unidentified species of labelled DNA which sedimented at 40 to 100S, between the main viral and cell DNA peaks (Doerfler *et al.*, 1974).

Therefore no convincing data, either quantitative or qualitative, on the integration of viral DNA during productive infection were available when the investigations described here were undertaken. These studies show that integration of adenovirus DNA occurs both in lytic and abortive infection, and that viral DNA replication is not necessary for integration in either type of infection. This is in agreement with a previous report (Doerfler, 1970) that the DNA of Ad12 integrated in abortively infected hamster cells in which viral DNA replication does not occur and was further ruled out by addition of cytosine arabinoside. From the time-course of integration during lytic infection (Chapter 2) it also appears that integration is not necessary for viral DNA replication, since viral DNA integrates mainly at late times after infection, when viral DNA synthesis is largely completed.

The present investigations on the integration of adenovirus DNA during lytic and abortive infection were undertaken with the aim of better understanding the meaning of integration in adenovirus-induced transformation. Evidence has been given that the viral DNA in adenovirus transformed cells is stably integrated into cellular DNA (Bellett, 1975; Green *et al.*, 1976). However, the role of integration in the transformation process cannot be defined until the viral and/or cellular functions responsible for it have been characterised.

The finding that integration of adenovirus DNA occurs during both lytic and abortive infection suggests that integration of viral DNA is not likely to be responsible for cell transformation. It appears that integration of viral DNA occurs by the same mechanism, or for the same reason, in both productively and abortively infected cells. This is likely to happen if integration is due to an active recombinational system in the cell, either pre-existing or activated by virus infection.

This would suggest that integration has no role during productive virus replication.

As mentioned previously, transformed cells arise from a minority (10^{-4} to 10^{-5}) of abortively infected cells. Following abortive infection by adenovirus, many cells die (Strohl, 1969a; 1969b; Younghusband, Tyndall, and Bellett, unpublished results). In mouse cells abortively infected by Ad5, viral DNA sequences representing different parts of the genome are equally and extensively integrated into the cell DNA. Surprisingly, none of 28 cloned but unselected survivors of Ad5 infection of mouse cells contained detectable viral DNA (H.B. Younghusband, personal communication). This type of experiment has not been done before, and indicates that most of the cells that integrate viral DNA die, while most of the survivors appear to contain no viral DNA sequences, or these are present in amounts undetectable with the viral probe used. Whether these surviving cells are 'cured' of viral DNA or they survived because they never contained extensive integrated viral DNA sequences, is not known. Hence, although integration of viral DNA is common in adenovirus infected cells, stable integration of substantial portions of the viral genome into the DNA of cells that subsequently survive and reproduce is relatively rare (less than 1/28 in mouse cells). This may contribute to the low frequency of transformation by adenoviruses.

Generally, adenovirus transformed cells, which are selected from the survivors of abortive infection as rare clones of epithelial cells able to grow in 0.1 mM Ca^{++}, contain only parts of the viral genome (Sharp *et al.*, 1974; Gallimore *et al.*, 1974; Flint *et al.*, 1976). In addition to the transforming viral sequences located within the extreme left 7 percent end of the adenovirus DNA (Graham *et al.*, 1974), other viral DNA sequences derived from every region of the viral genome may be present in varying proportions. Only Ad12 transformed hamster cells

(Fanning and Doerfler, 1976; Green *et al.*, 1976) and two lines of rat cells transformed by Ad5 ts125 at the non-permissive temperature (Mayer and Ginsberg, 1977) contain close to the full complement of the adenovirus genome. However, though present, late regions of the viral DNA are not expressed in transformed cells, and their presence appears to be irrelevant. For this reason it is very unlikely that the capacity of Ad5 ts125 to transform with increased frequency at the non-permissive temperature, is due to an increased extent of the viral DNA sequences integrated, as suggested by Mayer and Ginsberg (1977), since the only viral DNA sequences necessary for transformation are in the left-hand 7 percent of the viral DNA.

Mayer and Ginsberg (1977) also suggested that the ts125 gene product directly controlled transformation by modulating the extent of viral DNA integrated into the cell DNA. The results presented here on the integration of Ad5 and its mutants ts125 and ts36, suggest that the ts125 gene product is not involved in integration, and that it affects transformation frequency for some other reason. Also the fact that Ad5 ts36, which is transformation negative at the non-permissive temperature, integrates every portion of its DNA during abortive infection at the non-permissive temperature as well as at the permissive temperature, shows that the effect of this gene on transformation frequency is also not due to an effect on the frequency of integration of viral DNA during abortive infection.

In conclusion, all parts of the genome of adenovirus are integrated with the DNA of abortively infected cells, while most transformed cells contain only part of the viral DNA. This appears to be due to the fact that transformed cells are a selected population, while abortively infected cells represent a mixed population of cells which contain a

minority of cells destined to survive, and an even smaller proportion of potential transformants. Viral genes that influence the frequency of transformation do not appear to do so because of an effect on integration.

Chapter 5

MATERIALS AND METHODS

Cells and Media

Primary cultures of chick embryo kidney (CEK) cells were prepared as described by Laver *et al.* (1971). Human KB and KEH cells were grown in Eagle's medium supplemented with 10 percent bovine serum. Mouse cells were a continuous line of C57 Black mouse embryo cells (C57B1 J. M. cells) originally isolated by Dr J. T. May on a 3T3 schedule and since maintained as a continuous cell line.

Viruses and Virus Growth

CELO virus was grown in CEK cells, adenovirus type 5 was grown in KB cells. Virus was purified in CsCl density gradients as reported by Laver *et al.* (1971) and Younghusband and Bellett (1971), and titrated by the indirect immunofluorescence technique (Philipson, 1961). Virus titers are expressed as infectious units (i.u.) per cell. Ad5 ts36 and ts125 were kindly supplied by Dr W. C. Russell and Dr J. F. Williams. The mutants were grown in KB cells at 32-33°C and titrated in HEK cells at the same temperature.

Radioactive Isotopes

^{32}P (carrier-free) was obtained from the Australian Atomic Energy Commission, Lucas Heights, Sydney, and methyl-^{3}H-thymidine (48 Ci/mmol) and 2-^{3}H-adenosine (20 Ci/mmol) from the Radiochemical Centre, Amershan, U.K. Measurement of radioactivity was done as described by Younghusband and Bellett (1971).

Viral DNA

Viral DNA was prepared as described by Laver *et al.* (1971). For preparation of high specific activity ^{32}P-labelled CELO virus DNA, 1 mCi of ^{32}P was used for each 60 mm petri dish of infected CEK cells (2.5 ml

of phosphate free medium); high specific activity ^{32}P-labelled adenovirus type 5 DNA was obtained using 5 mCi of ^{32}P for each 75 mm^2 Falcon bottle of infected KB cells (equivalent to about three 60 mm petri dishes of infected cells). The specific activity of ^{32}P-CELO virus DNA ranged between 300,000 and 700,000 Cerenkov cpm per µg and that of Ad5 DNA between 1 million and 1.5 million Cerenkov cpm per µg.

Total Intracellular DNA

Confluent cell cultures either infected or uninfected were washed twice with phosphate buffered saline (PBS) and lysed by incubation for 2 h at 37°C with 1 ml per 60 mm petri dish of 1 mg/ml of pronase (preincubated 1 h at 36°C), 0.1 M NaCl, 5mM EDTA, 10mM Tris-HCl, pH 7.9, 0.5 percent SDS. The DNA was extracted twice with phenol saturated with 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.9 dialysed against 0.15 M NaCl 15mM sodium citrate, treated with ribonuclease (200 µg/ml) for 2 h at 37°C followed by pronase (500 µg/ml) for 30 min at 37°C, and extracted twice with chloroform-isoamyl alcohol (24:1). The DNA was then precipitated with ethanol and redissolved in STE (0.1 M NaCl, 50mM Tris-HCl, pH 7.4, 1mM EDTA). The concentration of DNA was determined by ultra violet (U.V.) spectrophotometry, assuming 1 absorbance unit at 260nm equals 50 µg/ml DNA.

Sedimentation of Intracellular ^{3}H-DNA in Alkaline Sucrose Gradients

(i) CELO virus-infected CEK cells

Primary chick cells were grown to confluence in medium containing ^{3}H-dThd (2-5 µCi/ml). The cells were then infected with CELO virus at a multiplicity of 1-23 i.u./cell and changed to fresh medium without ^{3}H-dThd. At the desired time post infection, the cell monolayers were washed with PBS and lysed for 1 h at room temperature with 1 ml/dish of

of 0.3 M NaOH, 0.5 percent SDS, 0.3 M NaCl, 3mM EDTA. The lysate was then gently poured onto the top of a preformed 35 ml 5 percent - 20 percent alkaline sucrose gradient (0.3 M NaOH, 0.7 M NaCl); each gradient was loaded with 2 ml of lysate, corresponding to approximately 7 x 10^6 cells. The gradients were centrifuged at 15°C for 2.5 h at 24,500 r.p.m. in a Spinco SW27 rotor. In some experiments the cells were lysed by a modification of the method of McGrath and Williams (1966), i.e., the cells were trypsinised, washed with PBS and layered directly on the top of an alkaline sucrose gradient between two layers of the alkaline lysing solution described above. About 7 x 10^6 cells, corresponding to the number of cells from two confluent cell monolayers in 60 mm petri dishes, were layered on each gradient, allowed to lyse at room temperature for 1 h and then centrifuged as above. ^{32}P-CELO virus DNA was added to the cell lysate prior to loading onto the gradient as a sedimentation marker. After centrifugation the gradients were fractionated from the bottom and a portion of each fraction was counted in a Packard Scintillation Spectrometer. The fractions corresponding to the viral and the cell DNA peaks were each pooled, neutralised with acetic acid, dialysed against 0.1 percent SDS in STE and concentrated by precipitation with 2 volumes of ethanol at -20°C or by dialysis against solid Carbowax (polyethylenglycol) 6000, and then dialysed against 0.1 percent SDS in STE. The amount of DNA was determined as above. In control experiments about 4 µg of unlabelled CELO virus DNA was added to each 60 mm petri dish of confluent uninfected CEK cells. The cells were then lysed and processed as described above. The total amount of viral DNA in each sample was determined by DNA-DNA reannealing kinetics as described below.

(ii) Ad5, Ad5 ts125 and Ad5 ts36-infected C57B1 J.M. mouse cells

C57B1 J.M. mouse cell cultures were prelabelled for 18-24 h with ^{3}H-dThd (0.5 - 1 µCi/ml) prior to infection. Semiconfluent cell monolayers (10^{6} - 2 x 10^{6} cells/60 mm petri dish) were then inoculated with Ad5 virus or its mutants ts36 or ts125 at a multiplicity of infection of 10 i.u./cell when virus was grown at 32.5 - 33°C and at a multiplicity of 50 i.u./cell when virus was grown at 39.9°C. The virus inoculum was allowed to adsorb to the cells for 1 h at room temperature; medium prewarmed at the appropriate temperature was then added to the cultures and incubation continued for 23-24 h at 39.9°C or for 70-72 h at 32.5°C. The cells were then washed and lysed. The lysate was centrifuged on alkaline sucrose gradients as described for CELO virus-infected CEK cells.

Each alkaline sucrose gradient was loaded with the lysate from two 60 mm dishes of cultured cells, equivalent to a maximum of 3 x 10^{6} cells. ^{32}P-labelled Ad5 DNA was added to the cell lysate, prior to loading on the gradients, as a sedimentation marker. However, ^{32}P-Ad5 DNA was never added to cells infected by either mutant virus at 39.9°C, since a very small amount of viral DNA is synthesised at this temperature. The contribution of the ^{32}P-viral DNA marker to the total amount of viral DNA produced at 32.5 - 33°C is negligible. After centrifugation, the gradients were processed as described above. In control experiments, from 4 to 10 µg of Ad5 DNA was added to each 60 mm dish of mock infected, ^{3}H-prelabelled mouse cells. The cells were then lysed and processed as described.

Sedimentation of 5-Bromo-2-deoxyuridine labelled intracellular ^{3}H-DNA in CsCl equilibrium gradients

CEK cells were grown to confluence in BHK Eagle's medium containing 10 µg/ml of 5-bromo-2-deoxyuridine (BUdR), 10 µg/ml of 2-deoxyuridine,

1 µg/ml of 5-Fluoro-deoxyuridine, and 5 µCi/ml of [2-^{3}H] Adenosine. BUdR was removed by thorough washing and the cells were then infected with CELO virus and incubated in medium containing 10^{-5}M thymidine. At various times post infection (usually 36 h) the cells were lysed as described above to obtain total intracellular DNA. BUdR labelled cells and DNA prepared from them were protected from light to minimise breakdown of the DNA. CsCl gradients were prepared by mixing solid CsCl with the DNA preparation and either neutral (10mM Tris-NCl, pH 7.4) or alkaline (0.1 N NaOH) solution to make up a 5 ml solution of density 1.76 g/ml for neutral CsCl or 1.81 g/ml for alkaline CsCl. ^{32}P-CELO virus DNA was added to each gradient before centrifugation as a marker. The samples were overlaid with paraffin oil and centrifuged in the Spinco Ti 50 rotor at 40,000 r.p.m. for 48-70 h. The gradients were then fractionated from the bottom and a portion (20 to 50 µl) of each fraction was counted in water soluble scintillation fluid. The fractions corresponding to the ^{3}H-cell DNA and the ^{32}P-viral DNA were pooled, dialysed against 0.1 percent SDS in STE, and the amounts of DNA were determined by U.V. spectrophotometry. In control experiments, uninfected ^{3}H-BUdR labelled CEK cells were used and unlabelled CELO virus DNA was added before lysis. The total viral DNA in each sample was estimated by DNA-DNA reannealing kinetics, as described below.

Analysis of Integration by the Network Technique

(i) Further analysis of fractions from alkaline sucrose gradients of CELO virus infected cells

^{3}H-cell DNA from alkaline sucrose gradients was ethanol precipitated, dissolved in 15mM NaCl and 1.5mM trisodium citrate, and further analysed by the network method (Varmus *et al.*, 1973). ^{3}H-DNA from infected cells was added at a final concentration of 6-8 µg/ml

(7 ng/ml viral DNA) to about 300 µg/ml of DNA extracted from uninfected cells. ^{32}P-CELO virus DNA was added to determine contamination of the cell DNA network by free viral DNA. The DNA sample was then denatured by heating at 100°C for 7 min, made 0.6 M in NaCl and allowed to reanneal to a Cot of 2-3 Mole sec/l at 68°C. The sample was then centrifuged in a Ti50 rotor at 40,000 r.p.m. for 15 min, the supernatant was removed and the visible pellet resuspended in 15mM NaCl, 1.5mM sodium citrate. The amounts of ^{3}H-cell DNA and free ^{32}P-viral DNA in the network and supernatant were estimated from radioactivity measurements and the amount of viral DNA in each was determined by reassociation kinetics. The amount of viral DNA integrated into the cell DNA was then calculated using an equation described by Bellett (1975).

(ii) Network analysis of total DNA from HEK cells infected with Ad5 ts36 or ts125

HEK monolayer cultures (15 to 20 cultures per sample, each about 2×10^7 cells) were inoculated with 3 PFU/cell to 6 PFU/cell of Ad5 ts36 or ts125 at either 32.5 - 33°C or 38.5°C. After 2 h, 20 ml medium was added to each culture and incubation continued for a further 16 h at the same temperature. The cells were washed twice with PBS, and total intracellular DNA was prepared as described above, except that the ethanol precipitation step was omitted. The samples were made 0.3 M in NaOH, left at room temperature for 15 min, and dialysed against 0.6 M NaCl, 0.05 M Tris pH 7.2, 1mM EDTA, 0.1 percent SDS. ^{32}P-Ad5 DNA was added to 5 ml of each sample to measure pelleting of free viral DNA and both portions of each sample were annealed to a Cot of 2 Mole sec/l. The network was pelleted at 140,000 g for 15 min in the MSE 8 x 50 ml rotor. The supernatant was again centrifuged. The combined network pellets from the two centrifugations were suspended in 10 ml of 0.3 M

NaOH. The portions containing ^{32}P-Ad5 DNA were counted and the remaining portions were boiled for 20 min, neutralised, precipitated with 2 volumes of ethanol at -20°C and redissolved in and dialysed against 0.01 M NaCl, 0.05 M Tris pH 7.2, 1 mM EDTA. The cell DNA concentration in each fraction was measured by optical density at 260nm, and the amount of viral DNA by reassociation kinetics as described below, using DNA from uninfected HEK cells as a control. The amount of integrated viral DNA was then calculated as before. The results were also corrected for recovery of DNA measured in parallel samples in which cell DNA was labelled with ^{3}H-dThd and ^{32}P-Ad5 DNA was added. The recoveries of cell and viral DNA were always similar, but recovery of DNA from the supernatant fractions (60 percent to 70 percent) was usually less than that from the network fractions (>90 percent).

DNA-DNA Reannealing Kinetics

The method of Pettersson and Sambrook (1973) was used. ^{32}P-labelled virus DNA, or restriction fragments of ^{32}P-labelled virus DNA, were sonicated with a Branson sonifer (Microtip) on setting 7 for a total of 1 minute. The sonicated ^{32}P-DNA was mixed with DNA purified from virus infected or uninfected cells as described above and calf thymus DNA was then added to a final total concentration of 800 µg/ml. The calf thymus, and the CEK or the mouse cell DNA had also been fragmented by sonication. The DNA samples were denatured by heating at 100°C for 10 min and allowed to reassociate in 1M NaCl at 68°C. Samples were taken at intervals diluted tenfold in 0.14 M phosphate buffer pH 6.8, at 0°C, and the fraction of ^{32}P-DNA that remained single-stranded was determined by chromatography on hydroxylapatite (Bio-Gel HTP) at 60°C. Radioactivity was determined by Cerenkov radiation. The reciprocal of the fraction of ^{32}P remaining single-stranded was plotted against time and the slopes of the lines were calculated by the least squares method. The ratio of the

slopes obtained for the reassociation of ^{32}P-viral DNA in the presence of DNA from infected cells plus calf thymus DNA and in the presence of calf thymus DNA alone was used to calculate the amount of viral DNA in the cell DNA (Gelb *et al.*, 1971).

Digestion of ^{32}P-Ad5 DNA with Restriction Endonucleases HpaI or HindIII

Restriction endonuclease HpaI was kindly donated by Dr K.D. Brown Restriction endonuclease HindIII was purchased from New England Biolabs. The HpaI enzyme reaction mixture contained: 10mM Tris-HCl, pH 7.9, 10mM $MgCl_2$, 10mM 2-mercaptoethanol, and the HindIII reaction mixture contained: 7mM Tris-HCl, pH 7.9, 7mM $MgCl_2$, 60mM NaCl. The amount of enzyme required for complete digestion was previously determined in trial reaction mixtures containing unlabelled Ad5 DNA. In every digestion of ^{32}P-Ad5 DNA at least twice the amount of enzyme required to digest that amount of DNA was used. Digestion was usually performed in a volume of 50-60 µl at 37°C for 1-2 h. Immediately after the digestion was completed, the digests were ice cooled and electrophoresed on agarose gel.

Gel Electrophoresis and Purification of ^{32}P-Ad5 DNA Restriction Fragments

Gel electrophoresis was done in 0.8 percent agarose (Sigma, electrophoresis grade) horizontal gels. The electrophoresis buffer contained 5mM sodium acetate, 40mM Tris-HCl, pH 8.2, 1mM EDTA. After electrophoresis, the DNA was visualised within the gel either by staining with ethidium bromide (0.5 µg/ml in electrophoresis buffer) for ½ h (Sharp *et al.*, 1973) or by autoradiography (Kodak RP X-Omat film). Ethidium bromide stained gels were photographed through a Kodak No.23 filter onto Polaroid film using ultraviolet illumination. Each of the ^{32}P-Ad5 DNA fragments was cut out of the agarose gel, dissolved in 5 M $NaClO_4$ at 60°C for ½ h, and loaded onto a 0.5 - 1 ml packed column of hydroxylapatite (Biogel, DNA grade) previously equilibrated with 5 M $NaClO_4$ (Daniell, 1976).

Each hydroxylapatite column was then washed in order, with 5 ml of 5 M $NaClO_4$, 5 ml of 0.14 M phosphate buffer, pH 6.8, and a total of 4 ml (1 ml first and then 3 ml) of 0.4 M phosphate buffer. 80 percent - 90 percent of the ^{32}P-DNA fragments were eluted as double stranded in the 0.4 M phosphate fraction. The 0.4 M phosphate fraction was then extensively dialysed against STE. The purity of each fragment was tested by electrophoresis of a small sample of each DNA fragment and, when necessary, fragments were further purified by electrophoresis as described above.

ADDENDUM

Radioactive profiles shown in the figures were normalised relative to peak height for ease of comparison. The radioactivity in these peaks was as follows:

Fig. 5: ^{3}H cpm in cell peak:(A): 4580; (B): 4059

^{32}P cpm in viral peak:(A): 1,000 (B): 1200

Fig. 7: (a) ^{3}H cpm in cell peak : 2900

^{32}P cpm in viral peak : 1900

(b) ^{3}H cpm in cell peak : 4000

^{32}P cpm in viral peak : 2,100

(c) ^{3}H cpm in cell peak : 1,800

^{32}P cpm in viral peak : 750

(d) ^{3}H cpm in cell peak : 2,500

^{32}P cpm in viral peak : 800

Fig. 8: ^{3}H cpm in cell peak (B):18,000; (A) 12,200

^{32}P cpm in viral peak (B): 2000; (A): 2500

Cell numbers referred to in the thesis were determined at the start of each experiment, and were not used to calculate viral DNA copies per cell. Copies per cell of viral DNA were obtained by dividing the number of viral DNA molecules by the number of diploid equivalents of cell DNA as described in the Legend to Table 1, and in Table 4 of Gelb, Kohne and Martin (1971). The amount of cell DNA was from A260 (corrected for hyperchromicity and recovery of ^{3}H as required). Because cell numbers increased during some experiments, and because the mouse cells used were subtetraploid, the number of diploid equivalents of cell DNA often exceeded the initial number of cells taken.

Sample calculation: Table 6 row 3 (Ad5 ts36 32.5°)

Ad5DNA = 3.8×10^{-11} µg, Diploid mouse DNA = 6×10^{-6} µg

$$0.16\mu g \text{ viral DNA} = \frac{0.16}{3.8 \times 10^{-11}} = 4.21 \times 10^{9} \text{ "copies"}$$

$$42.9\mu g \text{ cell DNA} = \frac{42.9}{6 \times 10^{-6}} = 7.15 \times 10^{6} \text{ (diploid) "cells"}$$

$$\text{Copies/cell viral DNA} = \frac{4.21 \times 10^{9}}{7.15 \times 10^{6}} = 588 \text{ copies/cell.}$$

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